PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/62, 1/19, C07K 14/47, C12Q 1/68, G01N 33/50 // C07K 14/72, 14/705

A1

(11) International Publication Number: WO 99/14344

(43) International Publication Date: 25 March 1999 (25.03.99)

(21) International Application Number: PCT/GB98/02759

(22) International Filing Date: 11 September 1998 (11.09.98)

(30) Priority Data:
9719496.3 13 September 1997 (13.09.97) GB

(71) Applicant *(for all designated States except US)*: GLAXO GROUP LIMITED [GB/GB]; Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).

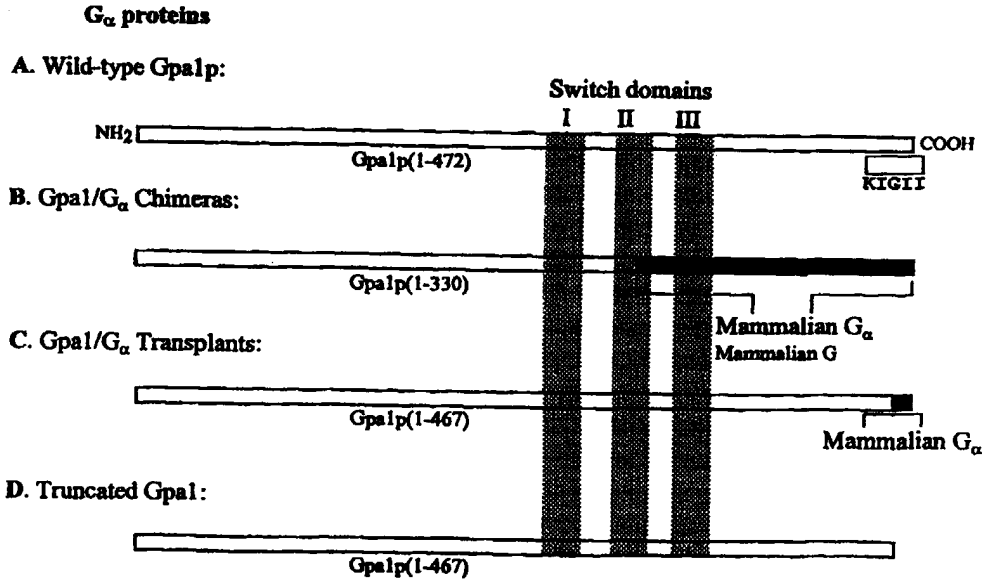
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BROWN, Andrew, James [GB/GB]; Glaxo Wellcome plc, Gunnels Wood Road, Stevenage, Herts SG1 2NY (GB). DOWELL, Simon, Jeremy [GB/GB]; Glaxo Wellcome plc, Gunnels Wood Road, Stevenage, Herts SG1 2NY (GB).

(74) Agent: LEAROYD, Stephanie, Anne; Glaxo Wellcome plc, Glaxo Wellcome House, Berkeley Avenue, Greenford, Middlesex UB6 0NN (GB).*

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: G PROTEIN CHIMERAS



(57) Abstract

A chimeric Gα protein having yeast Gα (Gpa1p) amino acid sequences modified by a minimum of 3 amino acids positions within the C–terminal 10 amino acids by substitution by alternative amino acids, a transformed yeast cell comprising said chimeric Gα protein and a method of screening for a compound able to interact with a receptor comprising contacting a compound of interest with said yeast cell and observing the growth response of the cell or observing production of a reporter gene product.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

G PROTEIN CHIMERAS

The present invention relates to chimeric G proteins, expression constructs therefor, yeast cells expressing such constructs and methods of making and using them.

G protein-coupled receptors are integral membrane proteins, characteristically with 7 transmembrane domains, which convey hormonal and sensory signals to the cell interior (see (1) for review). These receptors are commonly referred to as 7TM receptors or 7TMRs. The receptors respond to ligand binding by activating heterotrimeric G proteins composed of α, β and γ subunits. The G_α subunit is bound to GDP in the G protein trimer, and interaction with an activated (ligand bound) receptor induces the replacement of GDP with GTP. Accompanying conformational changes result in the dissociation of G_α-GTP and the G_β/G_γ particle, either of which can modulate ion channel or enzyme effectors to cause signal propagation. The signal persists until G_α, which has GTPase activity, hydrolyses the bound GTP, allowing reassembly of the heterotrimer. Members of the RGS (Regulator of G protein Signalling) protein family regulate signal duration by acting as GAPs (GTPase activating proteins) for the G_α subunit (38) (17). The G protein signalling system appears to be common to all eukaryotes.

A well-characterised example of the trimeric G protein signalling system is the pheromone response pathway of the budding yeast *Saccharomyces cerevisiae* (20). Cells of the *MAT***a** mating type express a receptor encoded by the *STE2* gene. This receptor becomes activated upon binding of the α-factor mating pheromone, a peptide secreted by cells of the opposite (*MAT*α) mating type. The yeast G protein is assembled from the products of the *GPA1* (G_α), *STE4* (G_β), and *STE18* (G_γ) genes. The G_β/G_γ (Ste4p/Ste18p) particle released upon activation of the Ste2p receptor conveys the signal to a mitogen-activated protein kinase (MAPK) module. This leads to activation of the cyclin-dependent kinase inhibitor Far1p, causing cell cycle arrest and transcriptional induction of a set of genes involved in the mating process, including *FUS1*. The pathway is desensitised by Sst2p, a member of the RGS family. Cells of the opposite mating type (*MAT*α) express a different receptor (Ste3p) and thereby respond to

the pheromone (a-factor) secreted by *MAT***a** cells; otherwise the signalling apparatus utilised in the two mating types is the same.

At present, at least 16 G_α subunits, 5 G_β subunits and 11 G_γ subunits have been identified in mammals, which can assemble a wide diversity of trimeric G proteins. On the basis of sequence homology, the G_α subunits fall into at least four families, related to $G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$, or $G_{\alpha 12}$. Typically, a given 7TM receptor activates only a single or small subset of G_α subunits. Thus even in cells which express multiple G_α subunits, signalling may be specific to particular G proteins and their downstream signalling pathways. A combination of approaches has defined several specific regions of the G_α subunit as key determinants of receptor/G protein specificity. These include regions in the N-terminus, the switch II to switch III regions (21) which are primarily responsible for binding G_β/G_γ, and particularly regions of the C-terminus. For example, a cluster of mutations occurring in the C-terminal region of G_α have been found to cause defects in receptor coupling ((29)(13) and references therein). Also, peptides modelled on the C-termini of $G_{\alpha t}$ (transducin) and $G_{\alpha i2}$ compete for binding to rhodopsin and the β-adrenergic receptor, respectively (9)(30)(35) and antibodies directed against the G_α C-terminus can also block interaction with receptors (6)(10)(36). The normal response to agonist stimulation of the adenosine A_1 receptor in cultured mammalian cells (e.g. COS cells) is the activation of $G_{\alpha i}$-family proteins, resulting in inhibition of adenylate cyclase. $G_{\alpha q}$-containing heterotrimers in contrast do not normally respond to A_1 activation. However, $G_{\alpha q}$ subunit can be induced to interact with the A_1 receptor by exchanging the C-terminal four amino acids of $G_{\alpha q}$ for the corresponding amino acids of $G_{\alpha i2}$ (7). Hence, phospholipase C (an effector of activated $G_{\alpha q}$) can be activated by adenosine A1 receptor agonists when signalling is mediated by a chimeric G_α subunit (7). The somatostatin SST_3 receptor is incompatible with $G_{\alpha s}$, but can be coupled to the activation of adenylate cyclase in COS cells by replacement of five C-terminal amino acids of $G_{\alpha s}$ with corresponding residues from either $G_{\alpha i2}$, which is known to interact with the SST_3 receptor, or from $G_{\alpha 16}$(18). $G_{\alpha 15}$ and $G_{\alpha 16}$ interact with a wide range of 7TM receptors (26), and are unusual in this respect. In crystal structures which have been solved for G_{i1} (41) and G_t (19) trimers, the G_α C-terminal tail lies on a flat, largely uncharged surface of the G protein trimer which also includes two lipid groups covalently attached to the G_α

N-terminus and the G_γ C-terminus. This surface is believed to face the membrane and to be involved in interactions with the intracellular loops of the 7TM receptor.

Several reports have demonstrated that the yeast G protein signalling system can be coupled to heterologously expressed mammalian G protein-coupled (7TM) receptors. Some receptors, including the rat somatostatin SST_2 receptor (33), and the rat adenosine A_{2a} receptor (34), can interact directly with the yeast G_α protein Gpa1p, whereas other receptors, including the human growth hormone releasing hormone receptor (GHRHR)(12), are incompatible with Gpa1p. In order to allow coupling of these receptors, the yeast G_α subunit can be deleted and the heterologous receptor co-expressed with a full-length mammalian G_α subunit. Alternatively, chimeric G_α subunits have been used in which the C-terminal domain (approx. one third of the peptide sequence) of Gpa1p is replaced with the equivalent region of a mammalian G_α subunit. See WO95/21925 (American Cyanamid Company) for both approaches. Chimeras, or other modified or heterologous G_α subunits must satisfy several criteria to be useful in a yeast coupling system. Most importantly, they must bind efficiently to yeast G_β/G_γ to prevent signalling in the absence of activated 7TM receptor, and they must effectively interact with agonist-bound activated receptors to be capable of signal propagation.

Such heterologous or chimeric G_α components can facilitate the coupling of a heterologous 7TM receptor to the yeast signalling system (the pheromone response pathway) so that the effects of ligands such as potential new drug molecules on the receptor can be observed in phenotypic responses of the yeast cells. For example, cells may be induced to grow, and/or to convert an indicator dye compound in response to receptor agonists by introducing reporter genes downstream in the signalling pathway. However, chimeric constructs such as those described in WO95/21925 commonly show reduced affinity for the yeast G protein $\beta\gamma$ subunit pair and show increased background signalling. Because of the specificity of a given receptor for one or a small number of the known G_α subunits, different G_α constructs have been required to demonstrate functional coupling activity with the majority of receptors tested.

The discovery of new drugs able to act via 7TM receptors requires screens of high efficiency, yet high specificity. Currently, the yeast coupling system is constrained by receptor/G protein specificity which imposes a requirement for a wide variety of G_α constructs to be tried for each receptor tested for coupling. For receptors which fail to couple, there is uncertainty as to whether failure is due to receptor/G protein incompatibility, or other reasons such as inappropriate receptor conformation, for example. It would be desirable to be able to couple a number of different receptors to the yeast G protein signalling pathway using a single G_α construct. This can be beneficial in the study of orphan receptors, of which nothing is known of receptor/G protein specificity. Alternatively, an array of G_α constructs could be used in the study of orphan receptors.

Surprisingly, we have found that certain chimeric G proteins, which we refer to as G protein "transplants" can be at least ten-fold more efficient than previously known chimeras in coupling mammalian 7TM receptors to the yeast mating pathway.

Accordingly, in a first aspect the present invention provides a chimeric G_α protein having yeast G_α (Gpa1p) amino acid sequences modified by a minimum of 3 amino acids positions within the C-terminal 10 amino acids by substitution with alternative amino acids. Preferably the chimera includes at least 5 such substitutions.

Accordingly, in a second aspect the present invention provides a chimeric G_α protein having yeast G_α (Gpa1p) amino acid sequences modified at a minimum of three amino acid positions within the C-terminal 10 amino acids by substitution with amino acids from a heterologous G_α protein. Preferably the chimera includes at least 5 substitutions. In preferred embodiments of this aspect of the invention, the chimera includes at least 3, desirably at least 5 consecutive amino acids corresponding to an amino acid sequence derived from the C-terminal 10 amino acids of a heterologous G_α protein. For example, the C-terminal 5 amino acids of a mammalian G_α protein, for example $G_{\alpha i6}$, but feasibly any G_α, may replace at least the C-terminal 5 amino acids of the endogenous yeast G_α protein to provide a chimera according to the invention. We have designated such chimeras "transplants" or "transplant chimeras".

The chimeric G_α proteins of the present invention are believed to adopt a conformation which is more compatible with signal transduction in yeast (and is presumed to be closer to the native yeast G_α conformation) than that of previously known chimeras in which the C-terminal domain of the yeast protein was exchanged for that of a mammalian G_α protein. Thus the chimeras of the present invention have the advantage of changing receptor specificity and permitting the coupling of diverse receptors to the yeast signalling pathway with only minimal changes to the Gpa1p sequence. As a result of the close similarity to wild-type Gpa1p, these chimeric G_α subunits retain a similar affinity for G_β/G_γ as Gpa1p, and expression can be directed by the *GPA1* promoter to achieve an optimal stoichiometry of G_α. Expression from various other promoters is also compatible with receptor coupling.

In a third aspect, the present invention also provides a nucleotide sequence encoding a chimeric G_α protein according to the present invention. Also provided is an expression vector comprising said nucleotide sequence and capable of expressing the nucleotide sequence on transfection into a suitable host cell. The construction of expression vectors including suitable promoters, transcription termination sequences and marker genes will be apparent to a person skilled in the art. The host cell may desirably be a yeast cell of the species *Saccharomyces cerevisiae* and represents a further aspect of the invention.

Accordingly, the invention also provides a transformed yeast cell comprising a nucleotide sequence which encodes a chimeric G_α protein according to the present invention, for example a heterologous G protein-coupled receptor including receptors for which the ligand is unknown, and a nucleotide sequence which encodes a chimeric G_α protein comprising yeast G_α (Gpa1p) amino acid sequences and at least 3 amino acids derived from the amino acid sequence of the C-terminal 10 amino acids of a G_α protein according to the present invention for example a heterologous G_α protein, such as the mammalian, $G_{\alpha 16}$ protein. The heterologous receptor may be a 7TM receptor. Such receptors include those for acetylcholine, adrenaline, noradrenaline, dopamine, histamine, melatonin, serotonin, angiotensin, prostaglandins, cannabinoids, neuropeptide

Y, substance P, opioids, glucagon, angiotensin, bradykinin, chemokines, thrombin, glycoprotein hormones, adenosine, nucleotides, and somatostatin.

Transformed yeast cells according to the present invention may also comprise a nucleotide sequence encoding a reporter gene operatively associated with a promoter responsive to the G protein signalling pathway. Such reporter genes may include *HIS3* or other auxotrophic markers (such as *URA3*, *LEU2*, or *TRP1*) or genes which confer resistance or sensitivity to drug selections, such as *CYH2* or *G418*^{R} or other genes such as those encoding intracellular enzymes such as β-galactosidase (*LacZ*) and luciferase, or green fluorescent protein (GFP), or genes encoding secreted enzymes such as a phosphatase such as *PHO5*, or a kinase. Desirably, yeast cells may contain combinations of multiple reporter genes, such as *FUS1-HIS3* and *FUS1-lacZ*. In preferred embodiments the transformed yeast cells will also include mutations in at least one of the *GPA1*, *SST2* or *STE2* genes. Preferably such mutations will be deletions. Desirably, *FAR1* is also deleted when a reporter gene is used to monitor activity in the G protein signalling pathway. This ensures that growth can continue even under conditions which activate the pheromone response pathway. In alternative embodiments of this invention, the *FAR1* gene may remain intact so that agonist stimulation causing activation of the signalling pathway may be monitored as resulting in growth arrest.

Preferred yeast strains will have deletions of *SST2* and *GPA1* (yeast G_α) - the former to prevent down-regulation of the signal by Sst2p activation of GTP-ase, the latter to prevent signal quenching, which occurs when G_α is present in stoichiometric excess to G_β/G_γ, due to rapid reassociation of the actively signalling G_β/G_γ moiety into the inactive heterotrimer.

We have designated the chimera between the N-terminal 467 amino acids of Gpa1p and the 5 C-terminal amino acids of $G_{\alpha 16}$ as the Gpa1-$G_{\alpha 16}$ transplant. Similarly, the chimera containing the C-terminal 5 amino acids of $G_{\alpha q}$ is designated as the Gpa1-$G_{\alpha q}$ transplant, and the chimera containing the C-terminal 5 amino acids of $G_{\alpha s}$ is designated as the Gpa1-$G_{\alpha s}$ transplant. In addition, the following transplants have also been constructed. They are identical to those described above, ie. they comprise the N-terminal 467 amino

acids of Gpa1p and the C-terminal 5 amino acids from a G_α protein as follows: Gpa1-$G_{\alpha 12}$, Gpa1-$G_{\alpha 13}$, Gpa1-$G_{\alpha 14}$, Gpa1-$G_{\alpha i1}$, Gpa1-$G_{\alpha i3}$, Gpa1-$G_{\alpha 0}$ and Gpa1-$G_{\alpha z}$.

A further transplant was prepared between the yeast G_α, Gpa1p and Gpa3, a G_α subunit from the yeast *Ustilago maydis*. This transplant is designated Gpa1-Gpa3. These transplants illustrate that by changing the 5 C-terminal amino acids of Gpa1p the specificity of the G_α subunit for receptors can be altered. Also the results indicate that the effectiveness of the "transplants" in coupling to foreign receptors in yeast is unexpectedly good compared to chimeric subunits with longer heterologous G_α regions.

In mammalian cells, the purinergic nucleotide $P2Y_2$ receptor is coupled to the activation of phospholipase Cβ (PLCβ) via $G_{\alpha q}$. We have found that the Gpa1-$G_{\alpha q}$ transplant substantially improves the weak response to agonist observed with wild-type Gpa1p. Similarly, coupling of the somatostatin SST_2 receptor achieved with the Gpa1-$G_{\alpha 16}$ transplant was enhanced ten-fold compared to either wild-type Gpa1p or the $G_{\alpha i/0}$ family chimeras, which is remarkable considering this receptor interacts with $G_{\alpha i/0}$ proteins in mammalian cells. Also the $5HT_{1A}$ receptor can interact with the Gpa1-$G_{\alpha 16}$ transplant, even though it fails to stimulate wild-type Gpa1p in MMY9 yeast cells. Minimal amino acid substitutions can confer on yeast Gpa1p the properties of a generic G protein ($G_{\alpha 16}$) which was not possible under previously described approaches to chimera construction. Therefore this invention presents for the first time the possibility of a system comprising a single G_α subunit and able to couple a wide variety of 7TM receptors.

Moreover, we have found that the approach of substituting the five C-terminal amino acids of Gpa1p to generate the transplants is widely applicable, in that we have generated transplants of representative members of all four G_α families: $G_{\alpha i}$, $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\alpha 12}$. This was not possible in previously described approaches to chimera construction. Furthermore, all of the transplants can be expressed from the promoter of the *GPA1* gene to achieve optimal stoichimetry for efficient coupling. This was not possible in previously described approaches to chimera construction, as some of these chimeras required expression from stronger

promoters, as in the case of Gpa1/$G_{\alpha s}$ (ref:12). Lastly, the pheromone response pathway is not activated in cells which express integrated versions of the transplants in the absence of activated receptors. This indicates that manipulations to the C-terminal amino acids do not interfere with the interaction with G_{β}/G_{γ}; this was not true with previously described approaches to chimera construction. Together, our data suggest that the approach of creating transplants will be applicable to any newly discovered mammalian G_{α}, or to G_{α} subunits not described herein ($G_{\alpha t}$ or $G_{\alpha olf}$) or to G_{α} subunits derived from any other metazoan species.

The invention will now be further described, by way of example and illustration and not of limitation, by the following experimental examples with the aid of figures in which:

Figure 1 shows agonist-dependent growth of the modified yeast (*S. cerevisiae*) strain MMY9. Cells expressing one of four human G protein-coupled receptors illustrate the ability of these receptors to interact with and activate the endogenous yeast G_{α} subunit (Gpa1p);

Figures 2 A-D show induction of the *FUS1-lacZ* reporter gene in response to receptor agonists using cells expressing receptor plus different modified G_{α} subunits;

Figure 3 shows that constructs which express a Gpa1/$G_{\alpha 16}$ chimera (containing a large C-terminal domain derived from $G_{\alpha 16}$) reduce *FUS1-HIS3* reporter gene expression to basal levels;

Figures 4 A-D show agonist-dependent activation of the *FUS1-lacZ* reporter gene by receptor agonists, mediated by Gpa1p modified at the C-terminal five residues (the transplants);

Figure 5 is a diagrammatic representation of G_{α} subunit constructs used in this study. The switch domains are shaded. Numbers in parenthesis refer to amino acid numbers of wild-type G_{α} subunits. Gpa1/G_{α} chimeras in B) contained C-

terminal regions (hatched) as follows: $G_{\alpha i1}$(212-354), $G_{\alpha i2}$ (213-355), $G_{\alpha i3}$ (212-354), $G_{\alpha 0}$ (213-354), $G_{\alpha 16}$ (221-374), $G_{\alpha s}$ (235-394), $G_{\alpha q}$ (211-353);

Figure 6 (a) to 6 (h) are diagrammatic representations of certain plasmid constructs used in this study (plasmid maps);

Figure 7 shows the activity of the *FUS1-lacZ* reporter gene in MMY9 *(STE2)* and MMY11 *(ΔSTE2)* cells transformed with pRS314-Gpa1 in response to incubation with the agonist, α-factor;

Figures 8 A-C show comparisons of isogenic yeast strains containing either episomal or integrated constructs expressing the transplants Gpa1-$G_{\alpha z}$ (Fig.8A), Gpa1-$G_{\alpha 13}$ (Fig.8B), and Gpa1-$G_{\alpha 12}$ (Fig.8C). Cells were deleted for the endogenous *STE2* gene but were transformed with a plasmid (Yep24-STE2) to express Ste2p to enable detection of *FUS1-lacZ* reporter gene activation in response to α-factor.

Materials and Methods:

Plasmids and Strains

Nucleic acid manipulations were carried out according to standard methods (24).

Receptor Expression Constructs

Receptor expression constructs were based on the high copy number episomal yeast-*E.coli* shuttle vectors pFL61 (27), YEp24, and pDT-PGK (see plasmid map of Fig. 6(A)), which is identical to pPGK reported by Kang *et al.* (14). Complementary DNA (cDNA) sequences encoding unmodified human G protein-coupled receptors were introduced into these vectors between the promoter and terminator regions of the *PGK1* gene, to confer strong constitutive expression in yeast cells. The somatostatin SST_2 receptor (Genbank accession M81830) was introduced into pFL61. The melatonin ML_{1B} (Genbank accession U25341), somatostatin SST_5 (Genbank accession L14865), serotonin $5HT_{1A}$ (Genbank

accession X13556), and serotonin $5HT_{1D}$ (Genbank accession M81589) receptors were introduced into pDT-PGK. The purinergic nucleotide $P2Y_1$ receptor (Genbank accession S81950) and the adenosine A_{2b} receptor (Genbank accession M97759) were also introduced into pDT-PGK. The purinergic nucleotide $P2Y_2$ receptor was also introduced into pDT-PGK. The sequence of the $P2Y_2$ receptor corresponded to that reported by Parr *et al.* (Genbank accession U07225) (32)(31) except codon 348 was GAA (Glu) and not GGA (Gly), potentially as a result of phylogenetic variation. The gene encoding the endogenous yeast α-factor receptor *STE2* was expressed from its own promoter using the construct Yep24-STE2.

G_α Expression Constructs

To create a construct for expression of sequences encoding G protein α subunits, a cassette consisting of the 1 Kb upstream region of the *GPA1* gene (the *GPA1* promoter), plus a multiple cloning site and the transcription terminator region of the *ADH1* gene (ADHt) were inserted into the centromeric plasmid pRS314 (Stratagene), generating pJW1 (Fig. 6 (b)).The *GPA1* open reading frame was inserted into the *Nru*I site of pJW1 to create the *GPA1* expression plasmid pRS314-GPA1 (Fig. 6 (c)).

Gpa1/$G\alpha_{j3}$ Chimera

Sequence encoding the chimeric G_α subunit Gpa1/$G_{\alpha i3}$ was derived from the plasmid pADC2-SCGi3. The chimera encoded by this plasmid is of a structure identical to that reported by Kang et al. (14) (with a *Bam*HI site in the switch II domain) except that it contains the C-terminus of $G_{\alpha i3}$ rather than $G_{\alpha i2}$. The Gpa1/$G_{\alpha i3}$ expression construct used in this study was created by PCR amplification using oligodeoxynucleotide primers to incorporate *Nco*I and *Not*I restriction enzyme sites adjacent to the open reading frame.

The sequence encoding the Gpa1/$G_{\alpha i3}$ chimera was inserted between the *Nco*I and *Not*I restriction enzyme sites of pJW1, to create the plasmid pRS314-Gpa1/$G_{\alpha i3}$ (Fig. 6 (d)). Constructs to express the Gpa1/$G_{\alpha o}$, Gpa1/$G_{\alpha i1}$, Gpa1/$G_{\alpha i2}$ Gpa1/$G_{\alpha s}$ Gpa1/$G_{\alpha q}$ and Gpa1/$G_{\alpha 16}$ chimeras were derived from

pRS314-Gpa1/$G_{\alpha i3}$ by replacing the $G_{\alpha i3}$-derived sequence (between the *Bam*HI and *Not*I restriction enzyme sites) with sequences encoding corresponding C-terminal regions of $G_{\alpha 0}$, $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha s}$, $G_{\alpha q}$ and $G_{\alpha 16}$. Plasmid maps of pRS314-Gpa1/$G_{\alpha 0}$, pRS314-Gpa1/$G_{\alpha i1}$, pRS314-Gpa1/$G_{\alpha i2}$, and pRS314-Gpa1/$G_{\alpha 16}$ are presented in Figs 6 (e), (f), (g) and (h), respectively.

G_{α} Transplants

The 'transplants', which had amino acid modifications the extreme C-terminus of Gpa1p, were generated in three steps. First, the *Afl*II site located in the *GPA1* promoter of pRS314-GPA1 was removed by blunt-ending with Klenow and religation. Next, codon 467 of *GPA1* was altered from AAA to AAG by site-directed mutagenesis, which was carried out using the Quik-change kit (Stratagene). This nucleotide change introduced an *Afl*II site without changing the encoded protein sequence. Finally, the *Afl*II/*Xho*I fragment was replaced with oligodeoxynucleotide linkers created by annealing the pairs of oligodexoynucleotides shown in Table 1.

Table1

Oligodeoxy-nucleotide	Sequence (5' to 3')	Transplant
Gqtop	TTAAGGAATACAACCTAGTTTGAATTCCG SEQ ID NO: 1	Gpa1/$G_{\alpha q}$
Gqbtm	TCGACGGAATTCAAACTAGGTTGTATTCC SEQ ID NO: 2	
Gstop	TTAAGCAATACGAACTATTGTGAATTCCG SEQ ID NO: 3	Gpa1/$G_{\alpha s}$
Gsbtm	TCGACGGAATTCACAATAGTTCGTATTGC SEQ ID NO: 4	
Gotop	TTAAGGGTTGTGGCTTGTACTGAATTCCG SEQ ID NO: 5	Gpa1/$G_{\alpha o}$
Gobtm	TCGACGGAATTCAGTACAAGCCACAACCC SEQ ID NO: 6	
Gi1top	TTAAGGATTGTGGTTTGTTTTGAATTCCG SEQ ID NO: 7	Gpa1/$G_{\alpha i1}$
Gi1btm	TCGACGGAATTCAAAACAAACCACAATCC SEQ ID NO: 8	
Gi3top	TTAAGGATGTGGTTTGTACTGAATTCCG SEQ ID NO: 9	Gpa1/$G_{\alpha i3}$
Gi3btm	TCGACGGAATTCAGTACAAACCACATTCC SEQ ID NO: 10	
Gztop	TTAAGTATATAGGCTTGTGTTGAATTCCG SEQ ID NO: 11	Gpa1/$G_{\alpha z}$
Gzbtm	TCGACGGAATTCAACACAAGCCTATATAC SEQ ID NO: 12	
G12top	TTAAGGATATTATGTTGCAATGAATTCCG SEQ ID NO: 13	Gpa1/$G_{\alpha 12}$

G12btm	TCGACGGAATTCATTGCAACATAATATCC SEQ ID NO: 14	
G13top G13btm	TTAAGCAATTGATGCTACAGTGAATTCCG SEQ ID NO: 15 TCGACGGAATTCACTGTAGCATCAATTGC SEQ ID NO: 16	Gpa1/$G_{\alpha 13}$
G14top G14btm	TTAAGGAATTTAACTTGGTTTGAATTCCG SEQ ID NO: 17 TCGACGGAATTCAAACCAAGTTAAATTCC SEQ ID NO: 18	Gpa1/$G_{\alpha 14}$
G16top G16btm	TTAAGGAAATTAACCTATTGTGAATTCCG SEQ ID NO: 19 TCGACGGAATTCACAATAGGTTAATTTCC SEQ ID NO: 20	Gpa1/$G_{\alpha 16}$
Stoptop Stopbtm	TTAAGTGAGCGGCCGCGAATTCCG SEQ ID NO: 21 TCGACGGAATTCGCGGCCGCTCAC SEQ ID NO: 22	[truncated Gpa1p]

The resulting plasmids encoded in-frame fusions between amino acids 1 to 467 of Gpa1p and the 5 C-terminal acids of mammalian G_α subunits (Fig.5C). A plasmid to express a truncated version of Gpap1 lacking the 5 C-terminal acids (Fig.5D) was created by introducing a stop codon at codon position 468 by insertion of linkers as above (Table 1).

Yeast Strains

The yeast strain MMY9 was created to study the functional interactions between 7 transmembrane helix receptors and G proteins. This strain was derived from the common laboratory strain W303-1A (genotype: *MATa his3 ade2 leu2 trp1 ura3 can1*). Activation of the pheromone response pathway was monitored by two reporter genes, *FUS1-HIS3* and *FUS1-lacZ* which were integrated into the *FUS1* and *leu2* loci, respectively. The *FAR1* gene was deleted by one-step gene replacement using a *far1Δ::URA3* DNA construct, so that growth continued even under conditions which activated the pheromone response pathway. The *SST2* gene was deleted by one-step gene replacement using a *sst2Δ::URA3* DNA construct to prevent down-regulation of G protein signalling by the GTPase-activating function encoded by this gene. After each of these manipulations, the *ura3* marker was recovered by transformation with a *ura3Δ* fragment consisting of the of the *URA3* gene with an internal 243bp (*Eco*RV to *Stu*I) deletion, followed by 5-fluoro-orotic acid selection. The chromosomal *GPA1* (G_α) gene

was deleted by one-step gene replacement using a *gpa1Δ::ADE2* DNA construct.

The yeast strain MMY11 was derived from MMY9 by one-step gene replacement using a *ste2Δ::G418*^{R} DNA construct, selecting for geneticin resistant colonies and confirming that resistant colonies failed to respond to the Ste2p agonist, α-factor.

Assays for Reporter Gene Expression

Agonists somatostatin (S-14), melatonin, serotonin, adenosine 5'-diphosphate (ADP) and uridine 5'-triphosphate (UTP) were obtained from Sigma. Alpha factor was synthesised by Peptide and Protein Research, Exeter, UK. 5'-N-Ethylcarboxamidoadenosine (NECA) was obtained from Research Biochemicals International.

Assay for *FUS1-HIS3* Expression. Reverse halo assays were carried out by growing MMY9 cells to early stationary phase ($OD_{600} \approx 4$) in liquid SC-glucose (2%) medium lacking tryptophan and uracil. A uniform layer of cells (5×10^7) was plated to 22.5cm by 22.5cm bioassay dishes (Nunc) in 100ml SC-glucose agar (1%) equilibrated to 50°C. This medium lacked tryptophan, uracil and histidine, and was supplemented with 10mM 3-aminotriazole, and buffered to pH 7.0 with 0.1M sodium phosphate. Antibiotic discs were placed on the solidified agar, and a volume of agonist solution (1-5μl) was applied to each disc. Plates were incubated at 30°C for 3 days.

Assays for *FUS1-lacZ* Expression. β-galactosidase activities in cell extracts were measured with two assays. In the first assay (ONPG assay), cell extracts were incubated with the substrate ONPG as described by (37). Units were defined as $(A_{420} \times 1000)/(OD_{600} \times t \times v)$ (25). In the chemiluminescent assay, cells were grown to late logarithmic phase and diluted to 0.02 OD_{600} in 100μl SC-WH medium in the presence or absence of 1 μM α-factor in 96-well microtitre plates. After incubation (30°C; 6 hours), 20μl of cells were removed and mixed with 20μl assay mix (125 mM sodium phosphate pH 7.5, 15 mM $MgSO_4$, 200 μM Galacton-Star β-galactosidase substrate (Tropix), 10% (v/v)

Sapphire II (Tropix), 1 U/µl oxalyticase (Enzogenetics)). After incubation (30°C; 1 hr) chemiluminescence was determined in a Top-count scintillation counter (Packard).

Assay for combined *FUS1-lacZ* and *FUS1-HIS3* Expression. *In vivo* assays of reporter gene induction (CPRG assays) were carried out by suspending cells to 0.02 OD_{600} in 200µl SC-glucose (2%) lacking tryptophan, uracil and histidine. This medium was supplemented with agonists, and additionally 10mM 3-aminotriazole and the β-galactosidase (*lacZ*) substrate chlorophenolred-β-D-galactopyranoside (CPRG; Boehringer) to a concentration of 0.1mg/ml. To visualise the colour change reaction, the medium was buffered to pH 7 with 0.1 M sodium phosphate. The assay was conducted in a 96-well microtitre plate format. Plates were incubated for 24 hours without agitation, and absorbance at 570nm was determined using a Victor microtitre plate reader (Wallac). EC_{50} values (+/- standard error) were estimated by curve-fitting, using the Robosage software package.

Yeast strains used in this study:

Strains	Genotype
W303-1A	*MAT***a** *his3 ade2 leu2 trp1 ura3 can1*
MMY9	W303-1A *fus1:FUS1-HIS3 FUS1-lacZ::LEU2 far1Δ::ura3Δ gpa1Δ::ADE2 sst2Δ::ura3Δ*
MMY11	MMY9 *ste2Δ::G418*^{R}

Experiment 1: Receptors activating yeast pheromone pathway

This experiment used four human G protein-coupled receptors as examples of receptors capable of activating the yeast pheromone response pathway. The four receptors were: the melatonin ML_{1B} receptor, the serotonin $5HT_{1A}$ receptor, the somatostatin SST_2 receptor and the purinergic nucleotide receptor, $P2Y_2$. These receptors were expressed in yeast strain MMY9 described above. This strain was deleted for *SST2, FAR1 and GPA1* but retained the endogenous α-factor receptor encoded by *STE2*. Activation of the pheromone response pathway was monitored with two integrated reporter genes: *FUS1-HIS1* allowing

a growth readout in medium lacking histidine, and *FUS1-lacZ* allowing a β-galactosidase readout. These assays are described herein above. We initially investigated signalling of these receptors via the endogenous yeast heterotrimeric G protein (Gpa1p/Ste4p/Ste18p). Receptors were expressed in yeast strain MMY9 from the strong *PGK* promoter, using high copy number episomal vectors. In Fig. 1, panels A-F show a reverse halo assay performed using MMY9 cells co-transformed with the plasmid pRS314-GPA1 (panels B to F), which expresses the wild-type *GPA1* gene and, in addition, with either a receptor expression construct or vector, as follows: pDT-PGK (vector; panel B); pFL61-SST_2 (panel C); pDT-PGK-ML_{1B} (panel D); pDT-PGK-$P2Y_2$ (panel E); pDT-PGK-5-HT_{1A} (panel F). Agonist-dependent activation of *FUS1-HIS3* was determined by reverse halo assay in which agonists were applied to the filter discs in the arrangement shown in panel A, in quantities as follows: 3 nmol of somatostatin-14 (S-14); 40 nmol of melatonin (Mel); 3 nmol of α-factor; 100 nmol of UTP; 40 nmol of serotonin (5-HT). As expected, treatment of MMY9 cells with the yeast pheromone, α-factor, stimulates the endogenous yeast receptor Ste2p, resulting in increased expression of *FUS1-HIS3* to enable a halo of cell growth in the absence of histidine (Fig. 1, Panel B). Similarly, MMY9 cells expressing the ML_{1B} receptor (panel D) activated *FUS1-HIS3* in response to melatonin, as well as in response to α-factor. Cells expressing the SST_2 receptor (panel C) responded to somatostatin, and cells expressing the $P2Y_2$ receptor (panel E) responded to the agonist UTP. The zones of cell growth in Fig. 1 are comparable in size, although different molar quantities of agonist were required for each receptor. Thus, the ML_{1B}, SST_2 and $P2Y_2$ receptors can couple to the yeast pheromone response pathway, via activation of the endogenous G_α, Gpa1p. MMY9 cells transformed with the 5-HT_{1A} receptor expression construct did not activate *FUS1-HIS3* in response to the agonist serotonin, suggesting that this receptor interacts poorly or cannot functionally interact with Gpa1p.

Experiment 2: Yeast/Mammalian Chimera coupling to receptors

This experiment illustrates that, for certain receptors, the efficiency of coupling to the yeast pheromone response pathway can be enhanced by creating chimeras between yeast and mammalian G_α subunits. A series of chimeric G_α subunits was generated in which the C-terminal domain (C-terminal 142 amino

acids) of Gpa1p was replaced with corresponding regions of rat $G_{\alpha 0}$, $G_{\alpha i1}$, $G_{\alpha i2}$ and $G_{\alpha i3}$ (Fig. 5(b)). To achieve an appropriate stoichiometry of G protein subunits, the chimeras were encoded on centromeric plasmids and expressed from the promoter of the *GPA1* gene. This avoids quenching of the G_{β}/G_{γ}-mediated signal, due to excess G_{α} (33). These constructs were used in an experiment in which MMY9 cells were cotransformed with pairs of plasmids, one expressing a G_{α} subunit and the second either vector (pDT-PGK) or one of the receptor expression constructs pFL61-SST_2, pDT-PGK-ML_{1B}, pDT-PGK-P2Y2 or pDT-PGK-5-HT_{1A}. Agonist-dependent activation of *FUS1-lacZ* was determined by incubating cells in medium supplemented with the chromogenic, cell-permeant β-galacosidase (*lacZ*) substrate, chlorophenolred-β-D-galactopyranoside (CPRG; Boehringer Mannheim). The extent of conversion of this substrate after 24 hr incubation at 30°C was determined by spectrophotometry and the results for the yeast G_{α} and the four chimeras are shown in Fig 2 A-D: 2A Ste2p receptor (pDT-PGK - transformed cells); 2B SST_2 receptor (pFL61-SST_2); 2C ML_{1B} receptor (pDT-PGK- ML_{1B}); 2D 5-HT_{1A} receptor (pDT-PGK-5-HT_{1A}).

As expected, α-factor stimulated the endogenous Ste2p receptor resulting in induction of *FUS1-lacZ* and provoking the strongest response with Gpa1p (EC_{50}; 5.2 +/- 0.4 nM). This value is consistent with the reported affinity of Ste2p for α-factor peptide (K_d; 17 nM) (3). MMY9 cells expressing the Gpa1/$G_{\alpha 0}$ and Gpa1/$G_{\alpha i2}$ chimeras also induced *FUS1-lacZ* in response to high concentrations of α-factor, but dose-response curves were displaced rightwards by at least two log units, suggesting that the Ste2p receptor interacts less efficiently with these chimeras than with wild-type Gpa1p. MMY9 cells expressing Gpa1/$G_{\alpha i1}$ or Gpa1/$G_{\alpha i3}$ chimeras did not induce *FUS1-lacZ* in response to α-factor. In contrast, the somatostatin SST_2 receptor was coupled to the pheromone response pathway by all the $G_{\alpha i}$ chimeras (EC_{50} values: 430 +/- 44 nM; 300 +/- 38 nM; 650 +/- 65 nM; 300 +/- 22 nM; and 630 +/- 30 nM for respectively Gpa1p, Gpa1/$G_{\alpha 0}$, Gpa1/$G_{\alpha i1}$, Gpa1/$G_{\alpha i2}$, and Gpa1/$G_{\alpha i3}$). This confirms that the chimeric G_{α} subunits are functional, and suggests that failure of receptors to couple to the pheromone response pathway is due to incompatibility with the G_{α} C-terminal region. We confirmed that the chimera proteins were produced at levels comparable to wild-type Gpa1p by quantitative Western blotting (data not

shown), using a polyclonal antibody directed against the N-terminus of Gpa1p which is common to all these proteins.

Of the other receptors, 5-HT_{1A} and ML_{1B} both exhibited more efficient coupling to the pheromone response pathway in cells expressing chimeric G_α subunits than those expressing Gpa1p. The 5-HT_{1A} receptor adopts a conformation in yeast capable of activating Gpa1/$G_{\alpha 0}$, supporting the hypothesis from Experiment 1 that this receptor is incompatible or poorly compatible with Gpa1p. Strikingly, the receptors are specific for particular chimeras, for example ML_{1B} activates Gpa1/$G_{\alpha i2}$ but not Gpa1/$G_{\alpha i3}$, even though these chimeras differ at just 14 amino acid positions, and levels of expression are similar in each case. When signalling was mediated by wild-type Gpa1p, *FUS1-lacZ* was induced to a lesser extent by $P2Y_2$ than the other receptors (data not shown), consistent with the requirement for greater molar quantities of agonist to give a similar zone of growth in the reverse halo assay (Fig. 1). The $P2Y_2$ agonist response was not enhanced by any of the $G_{\alpha i}$ chimeras (data not shown).

Experiment 3: Chimeras with C-terminal regions of $G_{\alpha 16}$

The $G_{\alpha 15}$/$G_{\alpha 16}$ subunits are reported to interact with a much broader range of receptors than is typical for a G_α subunit, and have the ability to couple receptors which normally interact with $G_{\alpha i}$, $G_{\alpha s}$ or $G_{\alpha q}$ (26). A Gpa1/$G_{\alpha 16}$ C-terminal domain chimera, if it exhibited similar properties to full-length $G_{\alpha 16}$, might be expected to couple diverse receptors to the pheromone response pathway. We constructed a plasmid encoding a Gpa1/$G_{\alpha 16}$ C-terminal domain chimera (pRS314- Gpa1/$G_{\alpha 16}$; Fig. 5(b); Fig. 6(h)) derived from the same centromeric vector as used for expression of functional chimeras described above. The level of *FUS1-LacZ* induction in MMY9 cells expressing the Gpa1/$G_{\alpha 16}$ chimera was determined by preparing cell extracts, and incubating them with the LacZ substrate o-Nitrophenyl β-D-galactopyranoside (ONPG; see Materials and Methods). Gpa1/$G_{\alpha 16}$ reduced *FUS1-LacZ* expression resulting in β-galactosidase activity of 79 +/- 8 units (Table 2). This level was intermediate between the activity of control cells transformed with vector (239 +/- 30 units), in which the pathway is constitutively activated due to the absence of G_α, and the basal activity in cells producing wild-type Gpa1p (19 +/- 4 units). Therefore, the

pheromone response pathway was partially activated, suggesting that this construct, which directs expression of Gpa1/$G_{\alpha 16}$ from the *GPA1* promoter, fails to sequester all free G_{β}/G_{γ}.

Table 2: Gpa1/$G_{\alpha 16}$ binds yeast G_{β}/G_{γ} but fails to support coupling of the Ste2p receptor.

G protein	Promoter	No Alpha Factor	Plus Alpha Factor	n
Vector		239 +/- 30	224 +/- 44	5
GPA1	*GPA1*	19 +/- 4	273 +/- 49	3
GPA1/G16	*GPA1*	79 +/- 8	79 +/- 4	3
GPA1/G16	*TEF1*	23 +/- 5	24 +/- 8	5
GPA1/G16	*GPD1*	16 +/- 4	13 +/- 4	3

We made further constructs to express Gpa1/$G_{\alpha 16}$ from the stronger *TEF1* and *GPD1* promoters. These reduced *FUS1-lacZ* expression to levels comparable to those in Gpa1p-producing cells (Table 1). In Fig. 3, MMY9 cells transformed with the different Gpa1/$G_{\alpha 16}$ expression constructs, or control plasmids were streaked to a non-selective agar plate supplemented with histidine (panel A) and to a selective agar plate lacking histidine and supplemented with 3-aminotriazole (panel B). The *TEF*-Gpa1/$G_{\alpha 16}$ and *GPD*-Gpa1/$G_{\alpha 16}$ expression constructs reduced *FUS1-HIS3* reporter gene expression to prevent growth on histidine-selective medium in common with the positive control plasmid (pRS314-GPA1), which expresses wild-type *GPA1*. In contrast, the Gpa1/$G_{\alpha 16}$ expression construct which utilised the *GPA1* promoter (pRS314-Gpa1/$G_{\alpha 16}$) failed to reduce *FUS1-HIS3* expression sufficiently to prevent growth under histidine-selective conditions, similar to the situation with vector-transformed cells (control). This result agrees with the ONPG assay results tabled above, therefore, the two reporter genes, *FUS1-lacZ* and *FUS1-HIS3*, behave similarly.

With none of the Gpa1/$G_{\alpha 16}$ expression constructs, however, did the Ste2p receptor appear to be coupled to the pheromone response pathway, because

treatment of cells with α-factor did not induce *FUS1-LacZ* (Table 1). MMY9 cells expressing the other receptors (ML_{1B}, $5\text{-}HT_{1A}$, $P2Y_2$, or SST_2) in combination with Gpa1/$G_{\alpha 16}$ also did not exhibit agonist-dependent induction of *FUS1-LacZ* (data not shown). Therefore, even though Gpa1/$G_{\alpha 16}$ can prevent the yeast G_β/G_γ particle from activating the pheromone response pathway, presumably by assembling into a G protein trimer, no free G_β/G_γ is released in the presence of activated receptors. Clearly, the approach of constructing this type of chimera, containing a large C-terminal domain derived from a mammalian G_α subunit fused to the N-terminal region of Gpa1p, is not generally applicable to all G_α subunits, but is successful with some G_α subunits (including the $G_{\alpha i}$ family and $G_{\alpha s}$) but not others (such as $G_{\alpha 16}$). The Gpa1/$G_{\alpha 16}$ chimera lacks the important property of full-length $G_{\alpha 16}$, that of coupling a much broader range of receptors than other G_α subunits. This experiment confirms that Gpa1/$G_{\alpha 16}$ C-terminal domain chimeric protein is produced in cells, and thus that its failure to couple G protein-coupled receptors to the pheromone response pathway is likely to be due to incompatibility between receptor and this G_α subunit.

A Gpa1/$G_{\alpha s}$ chimera has been reported to couple the growth hormone releasing hormone receptor (12) when expressed from the strong constitutive promoter of the yeast phosphoglycerate kinase (*PGK1)* gene. We constructed Gpa1/$G_{\alpha s}$ and Gpa1/$G_{\alpha q}$ chimeras similar to those descibed above (Fig.5B) and expressed them in MMY9 cells from the promoter of the *GPA1* gene. We assayed *FUS1-LacZ* activity in these cells using a chemiluminescent assay, rather than the ONPG assay described above. Results are presented in Table 3. As expected from the previous experiments, cells expressing Gpa1/$G_{\alpha 16}$ from the *GPA1* promoter exhibited significantly higher activities than control cells expressing Gpa1p. Gpa1/$G_{\alpha s}$ and Gpa1/$G_{\alpha q}$ chimeras are similar to Gpa1/$G_{\alpha 16}$ in that they also have significantly higher activities than control cells expressing Gpa1p. In contrast, cells expressing Gpa1/$G_{\alpha 0}$, Gpa1/$G_{\alpha i1}$, Gpa1/$G_{\alpha i2}$, or Gpa1/$G_{\alpha i3}$ from the *GPA1* promoter as expected contained activities comparable to control cells expressing Gpa1p. In this experiment only wild-type Gpa1p and the Gpa1/$G_{\alpha 0}$ chimera coupled the Ste2p receptor, as shown by increased *FUS1-LacZ* levels in cells incubated with 1 μM α-factor. This is consistent with the results of the CPRG assay above. This experiment further confirms that the Gpa1/$G_{\alpha 0}$, Gpa1/$G_{\alpha i1}$, Gpa1/$G_{\alpha i2}$, or Gpa1/$G_{\alpha i3}$ chimeras may be expressed from the *GPA1*

promoter and result in sufficiently low activation of the pheromone response pathway in the absence of activated receptor to be able to detect coupling, but that the Gpa1/$G_{\alpha s}$, Gpa1/$G_{\alpha q}$ and Gpa1/$G_{\alpha 16}$ chimeras may not.

Table 3: Activity of the pheromone response pathway in MMY9 cells expressing chimeric G_α subunits

G_α subunit	*FUS1-LacZ* Activity	
	No α-factor	1 μM α-factor
[vector]	8.7±2.0	7.4±1.6
Gpa1p	0.73±0.25	27.2±1.7
Gpa1/$G_{\alpha i1}$	0.71±0.2	0.68±0.15
Gpa1/$G_{\alpha i2}$	0.66±0.33	0.67±0.34
Gpa1/$G_{\alpha i3}$	0.50±0.22	0.76±0.23
Gpa1/$G_{\alpha 0}$	0.44±0.12	0.75±0.069
Gpa1/$G_{\alpha 16}$	3.6±1.9	3.6±1.5
Gpa1/$G_{\alpha s}$	3.1±2.0	5.0±3.0
Gpa1/$G_{\alpha q}$	8.2±3.9	9.6±4.1

Values are mean ± SD light units (x10^6 cps) in the chemiluminescent β-galactosidase assay. n ≥ 5.

Experiment 4: Transplant Approach

Fusions with longer N-terminal regions of Gpa1p and shorter regions of mammalian G_α subunits than the chimeras described above might still alter receptor specificity, given that minor modifications to the C-terminus of mammalian G_α subunits can change receptor specificity (7)(40)(23)(18). This could avoid the problem of chimeras having reduced affinity for G_β/G_γ. We created a refined set of Gpa1-G_α fusions, designated the "transplants" (to distinguish them from the chimeras having longer regions of mammalian G_α (Fig 5(B). These fusions had the five C-terminal residues (^{468}KIGII^{COOH}) of Gpa1p

replaced by the five C-terminal residues of $G_{\alpha 16}$ ($EINLL^{COOH}$), $G_{\alpha q}$ ($EYNLV^{COOH}$) or $G_{\alpha s}$ ($QYELL^{COOH}$)(see Fig. 5(C). MMY9 cells were co-transformed with the transplant constructs, and in addition with pDT-PGK (vector with no heterologous receptor) or the receptor expression constructs pDT-PGK-5-HT_{1A}, pFL61-SST_2 or pDT-PGK-$P2Y_2$. As in Experiment 2, agonist-dependent activation of *FUS1-LacZ* was determined by incubating cells in medium supplemented with CPRG, and the extent of conversion to product after 24 hr incubation at 30°C was determined by spectrophotometry. The results are depicted in Fig. 4 A-D. This experiment shows that the transplants were able to interact efficiently with G_β/G_γ, since they reduced *FUS1-LacZ* expression in MMY9 cells to basal levels when expressed from the *GPA1* promoter (Fig. 4A). In contrast to the C-terminal domain chimeras of Experiments 2 and 3, the Gpa1-$G_{\alpha 16}$, Gpa1-$G_{\alpha q}$ and Gpa1-$G_{\alpha s}$ transplants all retained the ability to couple the activated Ste2p receptor to the pheromone response pathway, although the dose-response curves suggested that compared to Gpa1p, the transplants interacted less efficiently with Ste2p (EC_{50} = 257 +/- 13 nM; 75 +/- 2 nM; 102 +/- 5 nM; respectively for Gpa1-$G_{\alpha s}$, Gpa1-$G_{\alpha 16}$ and Gpa1-$G_{\alpha q}$)(Fig. 4A). With human receptors as opposed to the yeast receptor, the transplants improved G_α subunit/receptor interactions. The Gpa1-$G_{\alpha q}$ transplant significantly enhanced coupling of the $P2Y_2$ receptor relative to Gpa1p, consistent with the finding that $P2Y_2$ signals to its effector phospholipase Cβ via $G_{\alpha q}$ in mammalian cells. In contrast, the capacity of the Gpa1-$G_{\alpha s}$ transplant to couple $P2Y_2$ to the pheromone pathway was indistinguishable from that of Gpa1p.

The Gpa1-$G_{\alpha 16}$ transplant enhanced the agonist response of cells expressing three of the four human receptor (SST_2, 5-HT_{1A}, and $P2Y_2$ but not ML_{1B}). MMY9 cells coexpressing the Gpa1-$G_{\alpha 16}$ transplant with the SST_2 receptor required roughly ten-fold less somatostatin to elicit a similar response to control cells producing Gpa1p (EC_{50} = 27 +/- 0.8 nM for Gpa1-$G_{\alpha 16}$; EC_{50} = 430 +/- 44 nM for Gpa1p). The 5-HT_{1A} receptor failed to signal in MMY9 cells through wild-type Gpa1p, but did so moderately well through the Gpa1-$G_{\alpha 16}$ transplant. For the SST_2 receptor, the Gpa1-$G_{\alpha 16}$ transplant was the most efficient G_α; for the 5-HT_{1A} and $P2Y_2$ receptors, the Gpa1-$G_{\alpha 16}$ transplant could support coupling but

not as efficiently as the Gpa1/$G_{\alpha 0}$ chimera and the Gpa1-$G_{\alpha q}$ transplant, respectively.

It has been reported (34) that deletion of the *STE2* gene encoding the Ste2p α-factor receptor can result in enhanced coupling efficiencies of heterologously expressed receptors. This may be due to competition between the receptors for G-protein trimers. To investigate the effect of receptor competition on the function of receptors and G_α subunits described herein, we constructed a derivative of MMY9 in which the the *STE2* gene was deleted by integrative disruption with the $G418^R$ resistance marker. The new strain was designated MMY11. This strain was used in an experiment in which MMY9 and MMY11 cells were transformed with the Gpa1p expression construct pRS314-GPA1 (Fig. 6C) and incubated with varying concentrations of α-factor in a CPRG assay (Fig.7). As expected, MMY11 fails to respond to α-factor.

MMY11 was used in an experiment in which cells were transformed with pairs of plasmids as before, one to express a G_α subunit and the other a receptor expression construct. This experiment involved the four receptor expression constructs previously described herein (pFL61-SST_2, pDT-PGK-ML_{1B}, pDT-PGK-$P2Y_2$ and pDT-PGK-5-HT_{1A}) and four further receptor expression constructs: pDT-PGK-SST_5, pDT-PGK-A_{2b}, pDT-PGK-$P2Y_1$ and pDT-PGK-5-HT_{1D}. We constructed futher transplant constructs to express modified versions of Gpa1p in which the C-terminal five amino acids were replaced with those of $G_{\alpha o}$ ($GCGLY^{COOH}$), $G_{\alpha i1}$ ($DCGLF^{COOH}$), $G_{\alpha i3}$ ($ECGLY^{COOH}$), and $G_{\alpha 14}$ ($EFNLV^{COOH}$). These were used in this experiment, along with the G_α subunit expression constructs previously described herein. Cells were subjected to CPRG assays to determine the concentration response curve to agonist for each combination of receptor and G_α subunit. Where pheromone response pathway activation was detected, the agonist concentration required to yield a half-maximal response (EC_{50} +/- standard error; all values in nM except where otherwise indicated) was determined by curve-fitting, and is shown in Table 4A. Where weak activation insufficient to carry out curve-fitting was detected, this is indicated in Table 4A (weak). Where no coupling was detected, this is also indicated (NC).

For comparison, Table 4B presents similar data obtained in MMY9; thus differences between the values in Tables 4A and 4B are likely to be due to the effect of Ste2p on signalling by heterologously expressed receptors.

5

Table 4A (MMY11):

	chimeras				transplants						
	Gpa1 p	Gpa1/ $G_{\alpha o}$	Gpa1/ $G_{\alpha i1}$	Gpa1/ $G_{\alpha i2}$	Gpa1/ $G_{\alpha i3}$	Gpa1/ $G_{\alpha q}$	Gpa1/ $G_{\alpha o}$	Gpa1/ $G_{\alpha i1}$	Gpa1/ $G_{\alpha i3}$	Gpa1/ $G_{\alpha s}$	Gpa1/ $G_{\alpha 14}$	Gpa1/ $G_{\alpha 16}$
SST_2	68±2	46±2	91±6	30±2	47±1	weak	85±4	5.6±0.6	1.8±0.1	NC	49±0.5	7.0±0.6
SST_5	28±0.8	115±4	137±8	56±3	97±4	66±3	11±0.5	4.9±0.2	3.6±0.2	weak	19±0.5	3.8±0.1
5-HT_{1A}	weak	390±14	11±0.5μM	2.5±0.2 μM	7±0.5 μM	weak	1.0±0.1 μM	1.3±0.1 μM	1.1±0.05 μM	weak	1.7±0.1 μM	1.7±0.1 μM
5-HT_{1D}	NC	2.0±1.1 μM	3.4±1.6 μM	355±82	1.8±0.5 μM	NC	weak	227±20	135±12	NC	NC	weak
ML_{1B}	912±300	weak	weak	407±200	NC	NC	500±200	14±5	26±5	NC	NC	1.4±0.15 μM
$P2Y_1$	NC	NC	NC	NC	NC	27±14 μM	NC	weak	weak	NC	12±2.5 μM	NC
$P2Y_2$	weak	NC	NC	NC	NC	3.8±0.1 μM	18±2 μM	5.2±0.5 μM	2.0±0.2 μM	weak	3.3±0.08 μM	4.84±0.0 6μM
$A2_B$	43±2	NC	NC	NC	NC	197±8	51±2.5	16±0.8	87±8	30±2.3	58±1.9	40±3

Values in nM except where otherwise indicated.

Table 4B (MMY9):

	chimeras				transplants						
	Gpa1 p	Gpa1/ $G_{\alpha o}$	Gpa1/ $G_{\alpha i1}$	Gpa1/ $G_{\alpha i2}$	Gpa1/ $G_{\alpha i3}$	Gpa1/ $G_{\alpha q}$	Gpa1/ $G_{\alpha o}$	Gpa1/ $G_{\alpha i1}$	Gpa1/ $G_{\alpha i3}$	Gpa1/ $G_{\alpha s}$	Gpa1/ $G_{\alpha 14}$	Gpa1/ $G_{\alpha 16}$
Ste2p	5.2±0.4	2±0.1μM	weak	21±2 μM	NC	102±5	31±4	87±1.5	14±2	257±13	288±50	75±2
SST_2	430±44	300±38	650±65	300±22	630±30	weak	ND	ND	ND	NC	ND	27±0.8
5-HT_{1A}	NC	2.6±0.4 μM	weak	weak	weak	weak	ND	ND	ND	weak	ND	weak
ML-1B	5.3±0.3 μM	weak	weak	1.4±0.2 μM	NC	NC	ND	ND	ND	NC	ND	weak
$P2Y_2$	weak	NC	NC	NC	NC	3.6±0.4 μM	ND	ND	ND	weak	ND	16±0.6 μM

Values in nM except where otherwise indicated. ND indicates not determined.

This experiment further illustrates that coupling efficiencies can be substantially enhanced by this "transplant" type of modification, even compared to the chimeric Gpa1/G_α subunits described in Experiments 2 and 3. Coupling of the Ste2p receptor was supported by all of the transplants, although none were as effective as wild-type Gpa1p, as indicated by the greater concentrations of α-factor required to elicit half-maximal responses (Table 4B). However, the transplants in general enhanced the coupling of human receptors, relative to wild-type Gpa1p (Table 4A) as indicated by the lower concentrations of agonists required to elicit half-maximal responses. All of the transplants enhanced $P2Y_2$ signalling except Gpa1-$G_{\alpha s}$, which was not significantly different from wild-type Gpa1p. Most efficient was the Gpa1-$G_{\alpha i3}$ transplant. Similarly, the transplants enhanced the efficiency of coupling of the SST_2, SST_5, ML_{1B}, $5HT_{1D}$ and A_{2b} receptors, relative to either Gpa1p or to the chimeras with long C-terminal domains of $G_{\alpha i/o}$, often with ten-fold or greater reductions in EC_{50}. The $P2Y_1$ receptor could be coupled to the pheromone response pathway only by transplants, with the Gpa1-$G_{\alpha 14}$ transplant being most efficient. It is informative to compare the Gpa1/$G_{\alpha i2}$ chimera and Gpa1-$G_{\alpha i1}$ transplant, which differ only in the length of sequence derived from the mammalian G_α (as the C-terminal five amino acids of $G_{\alpha i1}$ and $G_{\alpha i2}$ are identical). The EC_{50} values for ML_{1B} and SST_2 were more than ten-fold greater with the Gpa1-$G_{\alpha i1}$ transplant than with the Gpa1/$G_{\alpha i2}$ chimera. The same comparison can be made for the Gpa1/$G_{\alpha i3}$ chimera and Gpa1-$G_{\alpha i3}$ transplant. Clearly, chimeras with shorter lengths of mammalian G_α subunits can give more efficient coupling of some receptors. The only receptor in this study for which a transplant did not yield optimal coupling efficiency was $5HT_{1A}$ where the most efficient transplant was Gpa1-$G_{\alpha 0}$ (EC_{50} in MMY11; 1.0±0.1 μM) but the most efficient coupling was achieved with the Gpa1/$G_{\alpha 0}$ chimera (EC_{50} in MMY11; 390±14 nM). This suggests that for the majority of mammalian receptors, most efficient coupling in yeast will be achieved with the transplant type of G_α subunit.

Comparison of EC_{50} values obtained in MMY9 and MMY11 for identical combinations of receptor and G_α subunit indicates that the absence of Ste2p can enhance coupling efficiencies by aproximately 10-fold. Moreover, for certain poorly compatible receptor/G-protein combinations coupling could be detected in

MMY11 but not in MMY9, for example $5HT_{1A}$ and Gpa1p. This is fully consistent with the previously observed effects of deleting the *STE2* gene, reported by Price *et al.* (34), and does not affect the conclusions above.

We created a further construct to express a truncated Gpa1p molecule lacking the five C-terminal amino acids (Fig.8D). This truncation mutant has been reported to bind G_β/G_γ but fail to couple Ste2p to the pheromone response pathway (11). As expected, expression of this truncated Gpa1p mutant in MMY9 or MMY11 cells resulted in low levels of *FUS1-LacZ* activity, indicating the truncated mutant can bind G_β/G_γ (data not shown). In the CPRG β-galactosidase assay, this mutant fails to couple either Ste2p or other receptors to the pheromone response pathway (data not shown). This is fully consistent with the report of Hirsch *et al.* (11) and confirms the importance of the five C-terminal amino acids of the G_α subunit in the interaction with G-protein coupled receptors.

We made further transplant constructs to express modified versions of Gpa1p in which the C-terminal five amino acids were replaced with those of $G_{\alpha z}$ ($YIGLC^{COOH}$), $G_{\alpha 12}$ ($DIMLQ^{COOH}$) and $G_{\alpha 13}$ ($QLMLQ^{COOH}$). The Gpa1-$G_{\alpha z}$, Gpa1-$G_{\alpha 13}$ and to a lesser extent the Gpa1-$G_{\alpha 12}$ transplants expressed from the pRS314-Gpa1-$G_{\alpha z}$, pRS314-Gpa1-$G_{\alpha 13}$, and pRS314-Gpa1-$G_{\alpha 12}$ constructs were all able to couple the Ste2p receptor but they failed to fully sequester G_β/G_γ as they were associated with increased basal levels of *FUS1-LacZ* activity even in the absence of receptor activation (Fig.8). Conceivably, this might be due to disrupted interaction with G_β/G_γ, or to activation by an endogenous yeast protein, or reduced plasmid stability (see below). Quantitative immunoblotting suggested that all transplant proteins were produced to similar levels. We created further plasmids in which the Gpa1-$G_{\alpha z}$, Gpa1-$G_{\alpha 13}$ and Gpa1-$G_{\alpha 12}$ expression cassettes were contained in pRS304. The plasmid pRS304 is an integrating vector whereas pRS314 is an episomal, centromeric vector. The pRS304-Gpa1-$G_{\alpha z}$, pRS304-Gpa1-$G_{\alpha 13}$, and pRS304-Gpa1-$G_{\alpha 12}$ constructs were transformed into MMY11, targetting their integration into the *trp1* locus of this strain. The resultant strains expressed the Gpa1-$G_{\alpha z}$, Gpa1-$G_{\alpha 13}$ and Gpa1-$G_{\alpha 12}$ transplants from genes located chromosomally rather than from genes located on free, episomal plasmids. In the experiment presented in Figure

8, MMY11 cells were transformed with either pRS304-based or pRS314-based (integrating and episomal, respectively) plasmids to express Gpa1-$G_{\alpha z}$ (Fig.8A), Gpa1-$G_{\alpha 13}$ (Fig.8B), or Gpa1-$G_{\alpha 12}$ (Fig.8C) transplants. In addition, cells were transformed with Yep24-STE2 to express the *STE2* gene, as this had been deleted from the MMY11 strain. Activation of *FUS1-LacZ* in response to different concentrations of α-factor was determined in a CPRG assay. This experiment shows that integrating the transplant expression constructs into the chromosome reduces basal levels of *FUS1-LacZ* activity in the absence of receptor activation. This enables the Gpa1-$G_{\alpha z}$, Gpa1-$G_{\alpha 13}$ and Gpa1-$G_{\alpha 12}$ transplants to be tested in experiments with the human receptor expression constructs for their ability to couple heterologously expressed receptors (data not shown).

We carried out the same approach, of transferring the transplant expression cassette to a pRS304 plasmid and integrating into the genome of MMY11, for other transplants. This created a series of strains expressing chromosomal copies of the Gpa1-$G_{\alpha O}$, Gpa1-$G_{\alpha i1}$, Gpa1-$G_{\alpha i3}$, Gpa1-$G_{\alpha 14}$, Gpa1-$G_{\alpha S}$, Gpa1-$G_{\alpha q}$, Gpa1-$G_{\alpha 16}$ Gpa1-$G_{\alpha z}$, Gpa1-$G_{\alpha 13}$ and Gpa1-$G_{\alpha 12}$ transplants. In all cases, we observed the phenomenon described above, of reduced basal levels of *FUS1-LacZ* activity in the absence of receptor activation, without affecting EC_{50}. We conclude from this result that the episomal G_α subunits constructs are unstable. We postulate that in a population of cells containing an episomal G_α plasmid, a small proportion of cells may lose this plasmid. These cells would not be propagated as they lack the *URA3* gene. However, because they would lack any G_α subunit the pheromone response pathway would be constitutively activated and they would accumulate the LacZ enzyme. Thus elevated basal *FUS1-lacZ* levels would be apparent in the whole population, without changing apparent protein levels. With the integrated copy of the gene encoding G_α, this does not occur.

The set of strains containing the full range of integrated transplants represents the full diversity of known mammalian G_α subunits likely to be relevant to drug discovery ($G_{\alpha t}$ and $G_{\alpha olf}$ transplants have not been tested). The same approach of constructing transplants may be taken with any G_α subunits discovered in the future.

In conclusion, this approach represents a significant improvement over previously described technologies, because i) it is applicable to a wider range of G_α subunits, ii) it is applicable to $G_{\alpha16}$ in particular and iii) the Gpa1-$G_{\alpha16}$ transplant possesses, at least to some degree, the property of $G_{\alpha16}$ of interacting with a broad range of receptors, and therefore may be the G_α subunit of choice for coupling orphan receptors, for which the physiologically relevant G protein targets are unknown.

Bibliography

1. Baldwin, J. M. 1994. Current Opinion in Cell Biology **6**:180-190.

2. Bertin, B., et.al. 1992. Journal of Biological Chemistry **267**:8200-8206.

3. Blumer, K. J. and J. Thorner. 1990. Proc. Natl. Acad. Sci. U. S. A. **87**:4363-4367.

4. Bourne, H. R. 1997. Current Opinion in Cell Biology **9**:134-142.

5. Butkerait, P., et.al. 1995. Journal of Biological Chemistry **270**:18691-18699.

6. Cerione, R. A., et.al. 1988. J. Biol. Chem. **263**:9345-9352.

7. Conklin, B. R., et.al. 1993. Nature **363**:274-276.

8. Fargin, A., et.al. 1991. Cellular Signalling **3**:547-557.

9. Hamm, H. E., et.al. 1988. Science **241**:832-835.

10. Hamm, H. E., et.al. 1987. J. Biol. Chem. **262**:10831-10838.

11. Hirsch, J. P., et.al. 1991. Genes & Development **5**:467-474.

12. Kajkowski, E. M., et.al. 1997. Journal of Receptor & Signal Transduction Research **17**:293-303.

13. Kallal, L. and J. Kurjan. 1997. Molecular & Cellular Biology **17**:2897-2907.

14. Kang, Y. S., et.al. 1990. Molecular & Cellular Biology **10**:2582-2590.

15. Kleuss, C., et.al. 1993. Science **259**:832-834.

16. Kleuss, C., et.al. 1992. Science **358**:424-426.

17. Koelle, M. R. 1997. Current Opinion in Cell Biology **9**:143-147.

18. Komatsuzaki, K., et.al. 1997. FEBS Letters **406**:165-170.

19. Lambright, D. G., et.al. 1996. Nature **379**:311-319.

20. Leberer, E., et.al. 1997. Current Opinion in Genetics & Development **7**:59-66.

21. Lee, C. H., et.al. 1995. Molecular Pharmacology **47**:218-223.

22. Lichtarge, O., et.al. 1996. Proc. Natl. Acad. Sci. U. S. A. **93**:7507-7511.

23. Liu, J., et.al. 1995. Proc. Natl. Acad. Sci. U. S. A. **92**:11642-11646.

24. Maniatis, T., et.al.. 1982. Molecular Cloning, A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

25. Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

26. Milligan, G., et.al. 1996. Trends in Pharmacological Sciences **17**:235-237.

27. Minet, M., et.al.. 1992. Plant Journal **2**:417-422.

28. Oliner, J. D., et.al.. 1993. Nucleic. Acids. Res. **21**:5192-5197.

29. Onrust, R., et.al. 1997. Science **275**:381-384.

30. Palm, D., et.al. 1990. FEBS Lett. **261**:294-298.

31. Parr, C. E., et.al. 1994.. Proc. Natl. Acad. Sci **91**:3275-3279.

32. Parr, C. E., et.al. 1994. Proc. Natl. Acad. Sci. **91**:13067.

33. Price, L. A., et.al. 1995. Molecular & Cellular Biology **15**:6188-6195.

34. Price, L. A., et.al. 1996. Molecular Pharmacology **50**:829-837.

35. Rasenick, M. M., et.al. 1994. J. Biol. Chem. **269**:21519-21525.

36. Simonds, W. F., et.al.. 1989. Proc. Natl. Acad. Sci. U. S. A. **86**:7809-7813.

37. Slater, M. R. and E. A. Craig. 1987. Molecular & Cellular Biology **7**:1906-1916.

38. Tesmer, J. J., et.al. 1997. Cell **89**:251-261.

39. Van Dop, C., G. et.al. 1984. J. Biol. Chem. **259**:23-26.

40. Voyno-Yasenetskaya, T., et.al. 1994. J. Biol. Chem. **269**:4721-4724.

41. Wall, M. A.,et.al. 1995. Cell **83**:1047-1058.

CLAIMS

1. A chimeric G_α protein having yeast G_α (Gpa1p) amino acid sequences modified by a minimum of 3 amino acids positions within the C-terminal 10 amino acids by substitution by alternative amino acids

2. A chimeric G_α protein according to claim 1 wherein the amino acid sequences are modified by at least 5 amino acid positions within the C-terminal 10 amino acids by substitution by alternative amino acids.

3 A chimeric G_α protein having yeast G_α (Gpa1p) amino acid sequences modified at a minimum of three amino acid positions within the C-terminal 10 amino acids by substitution with amino acids from a heterologous G_α protein.

4. A chimeric G_α protein according to claim 3 wherein the amino acid sequence is modified by at least 5 amino acid positions within the C-terminal 10 amino acids by substitution with amino acids from a heterologous G_α protein.

5. A nucleotide sequence encoding a chimeric G_α protein according to any of claims 1 or 4.

6. A transformed yeast cell comprising a nucleotide sequence which encodes a heterologous G protein-coupled receptor and a nucleotide sequence according to claim 5.

7. A method of screening for a compound able to interact with a receptor comprising contacting a compound of interest with a cell according to claim 6 and observing the growth response of the cell.

8. A method according to claim 7 wherein the cell further comprises a reporter gene and the method includes the step of observing the production of a reporter gene product.

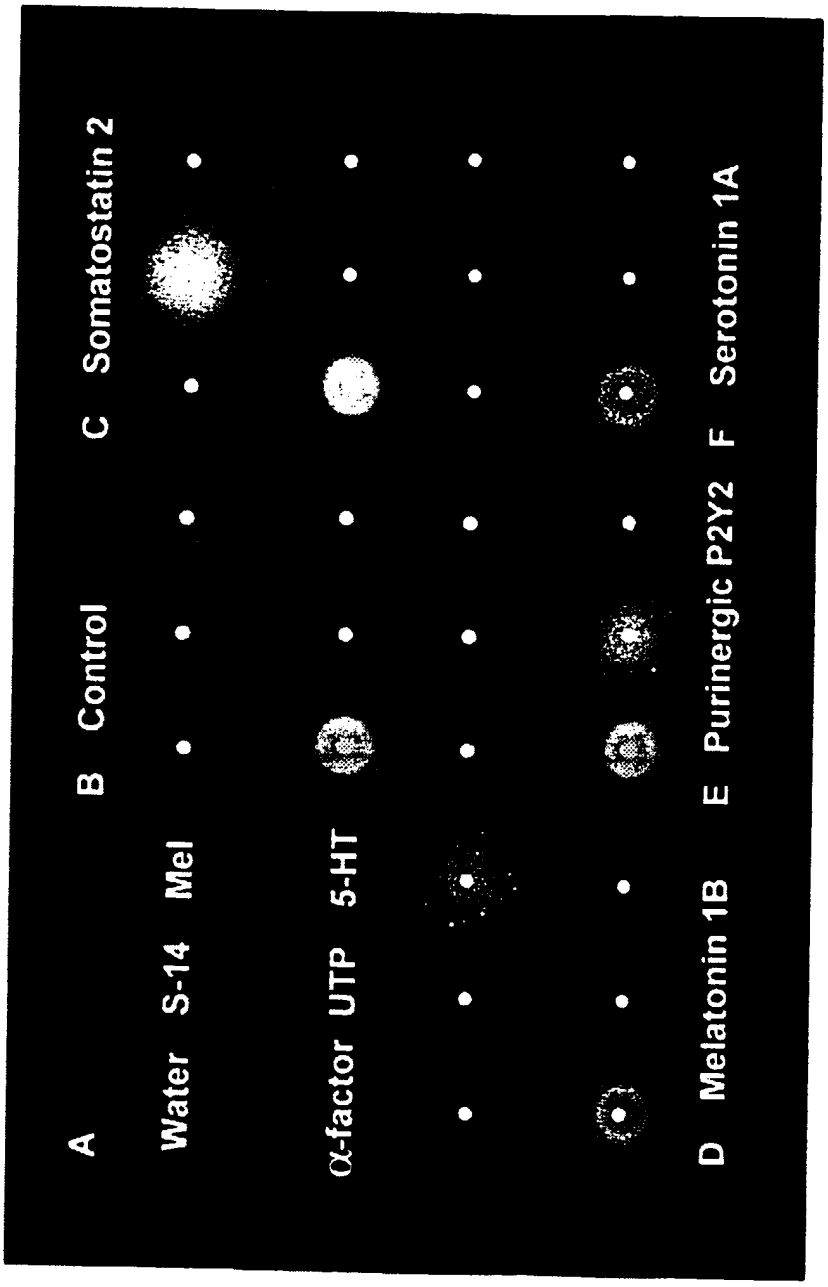

A
B Control
C Somatostatin 2
Water
S-14
Mel
α-factor
UTP
5-HT
D Melatonin 1B
E Purinergic P2Y2
F Serotonin 1A

FIG.1

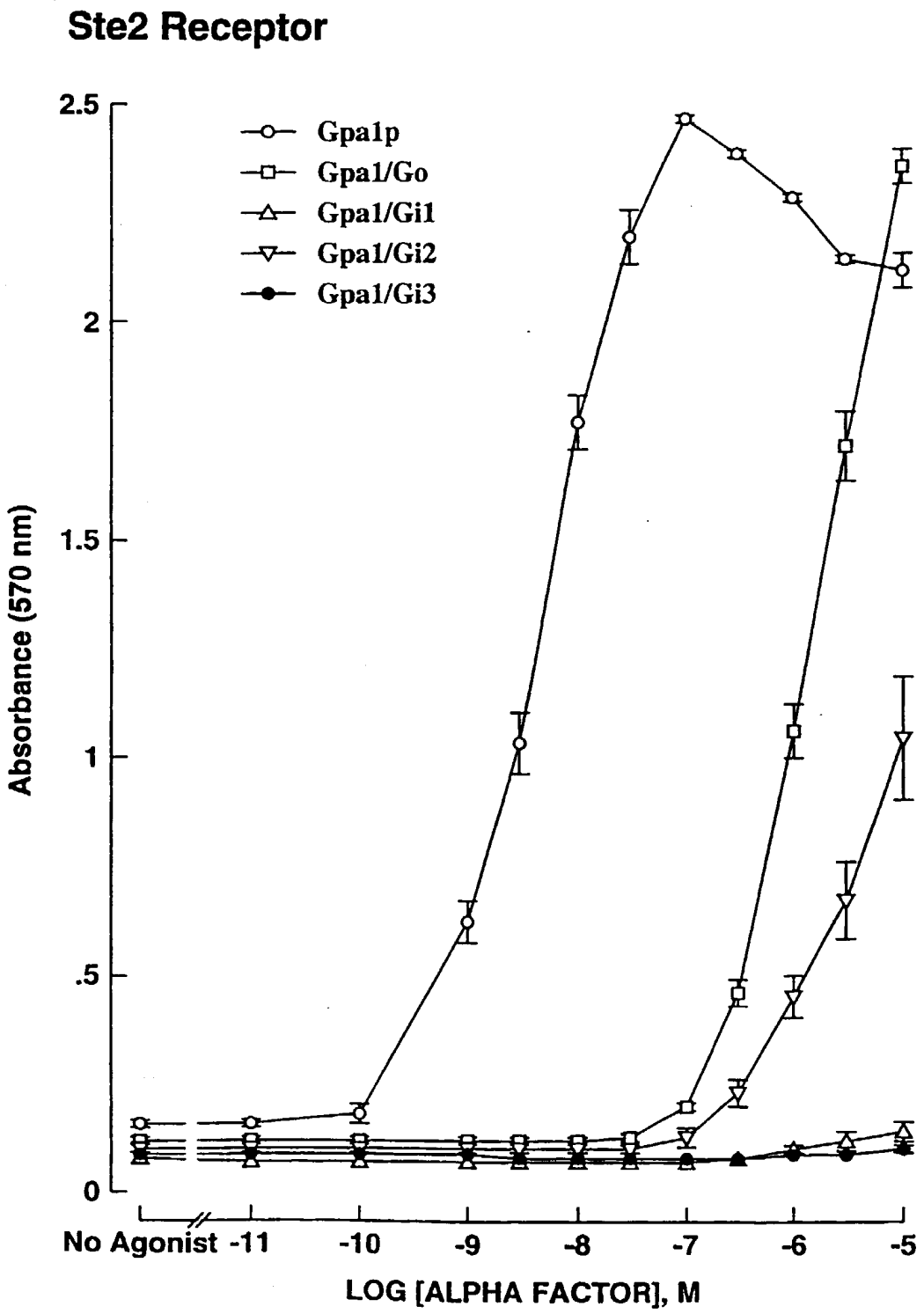

Ste2 Receptor
Gpa1p
Gpa1/Go
Gpa1/Gi1
Gpa1/Gi2
Gpa1/Gi3
Absorbance (570 nm)
2.5
2
1.5
1
.5
0
No Agonist
-11
-10
-9
-8
-7
-6
-5
LOG [ALPHA FACTOR], M

FIG.2(a)

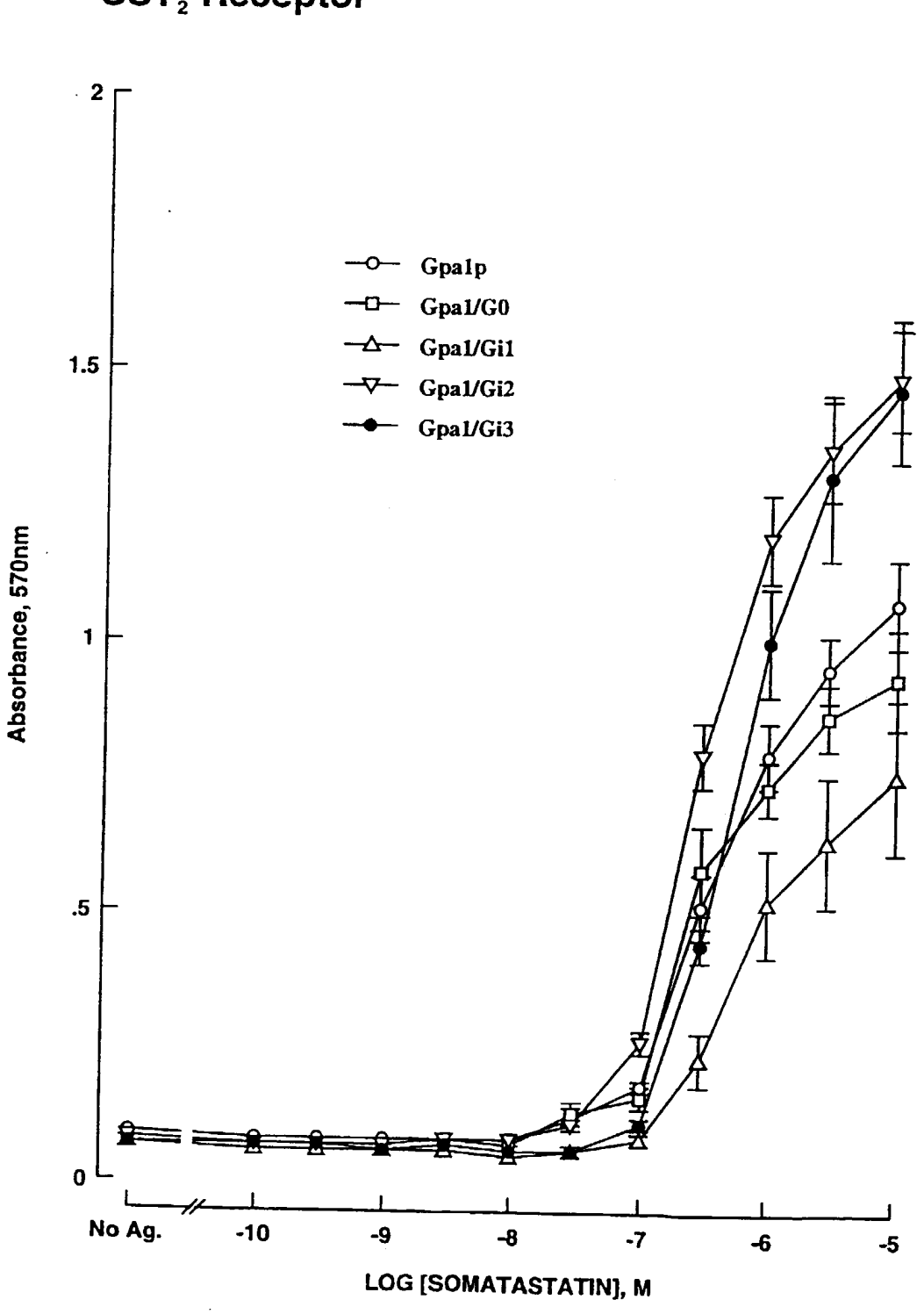

SST2 Receptor
Gpa1p
Gpa1/G0
Gpa1/Gi1
Gpa1/Gi2
Gpa1/Gi3
Absorbance, 570nm
2
1.5
1
.5
0
No Ag.
-10
-9
-8
-7
-6
-5
LOG [SOMATASTATIN], M

FIG.2(b)

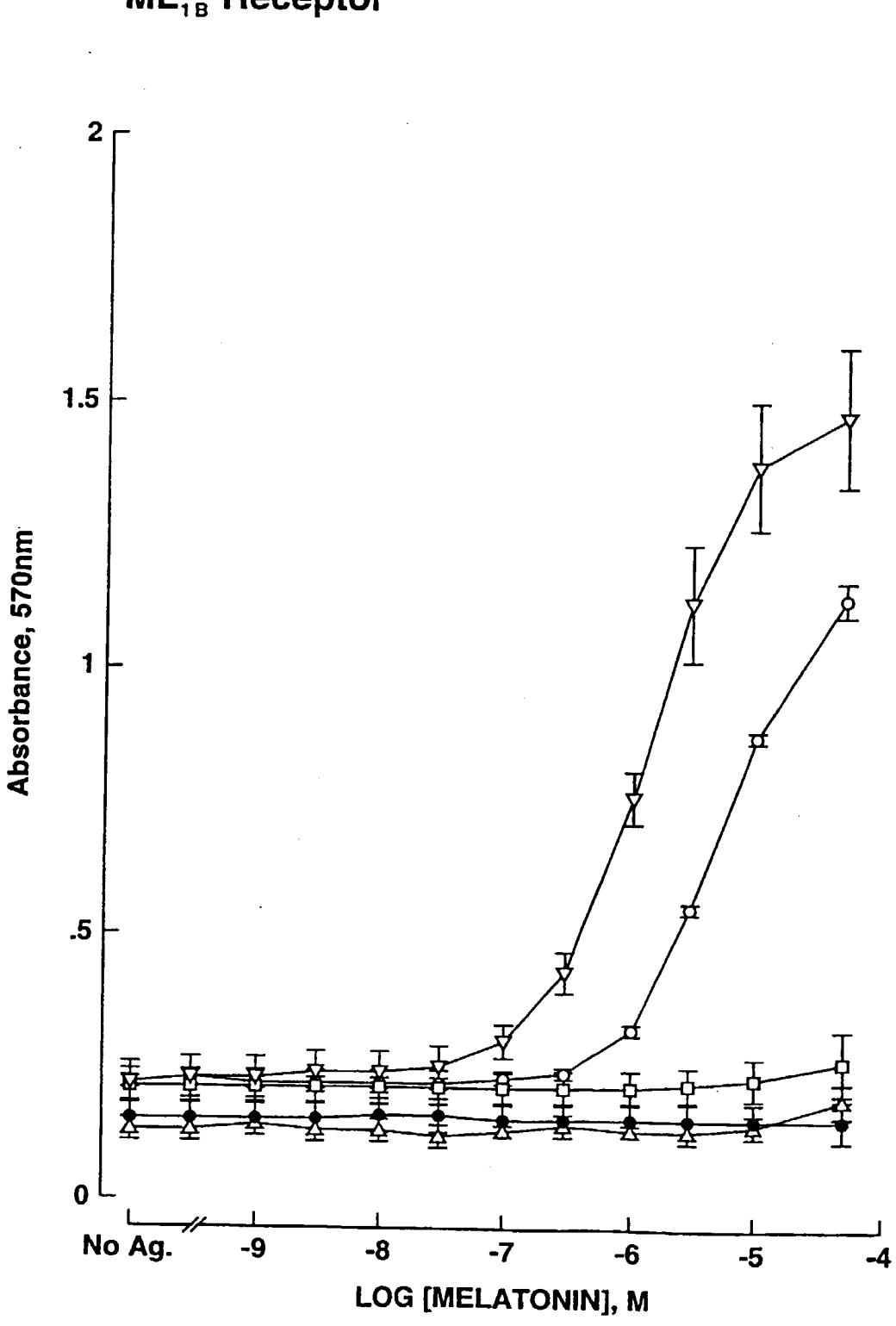

ML1B Receptor
Absorbance, 570nm
2
1.5
1
.5
0
No Ag.
-9
-8
-7
-6
-5
-4
LOG [MELATONIN], M

FIG. 2(c)

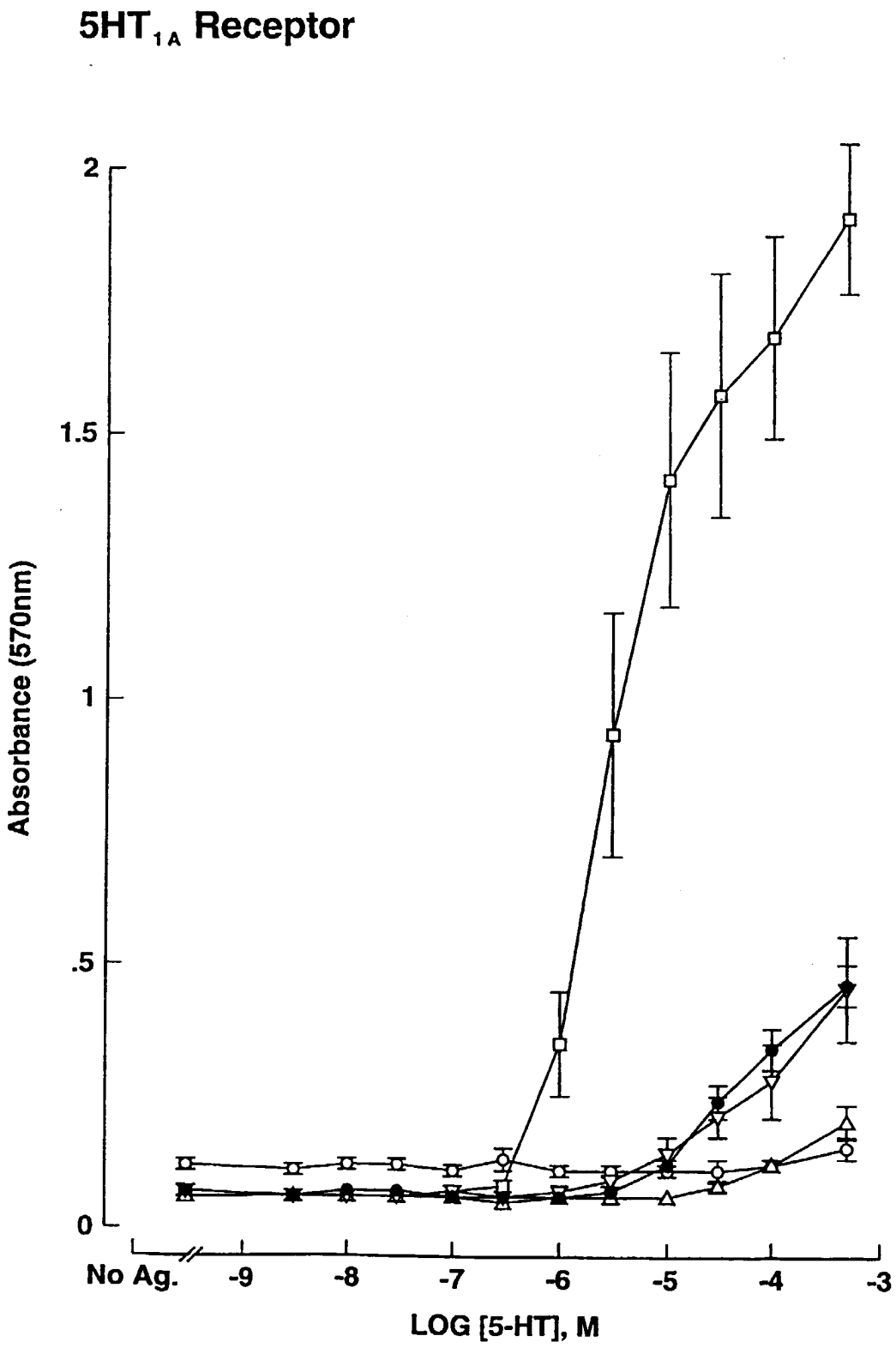

5HT1A Receptor
Absorbance (570nm)
2
1.5
1
.5
0
No Ag.
-9
-8
-7
-6
-5
-4
-3
LOG [5-HT], M

FIG.2(d)

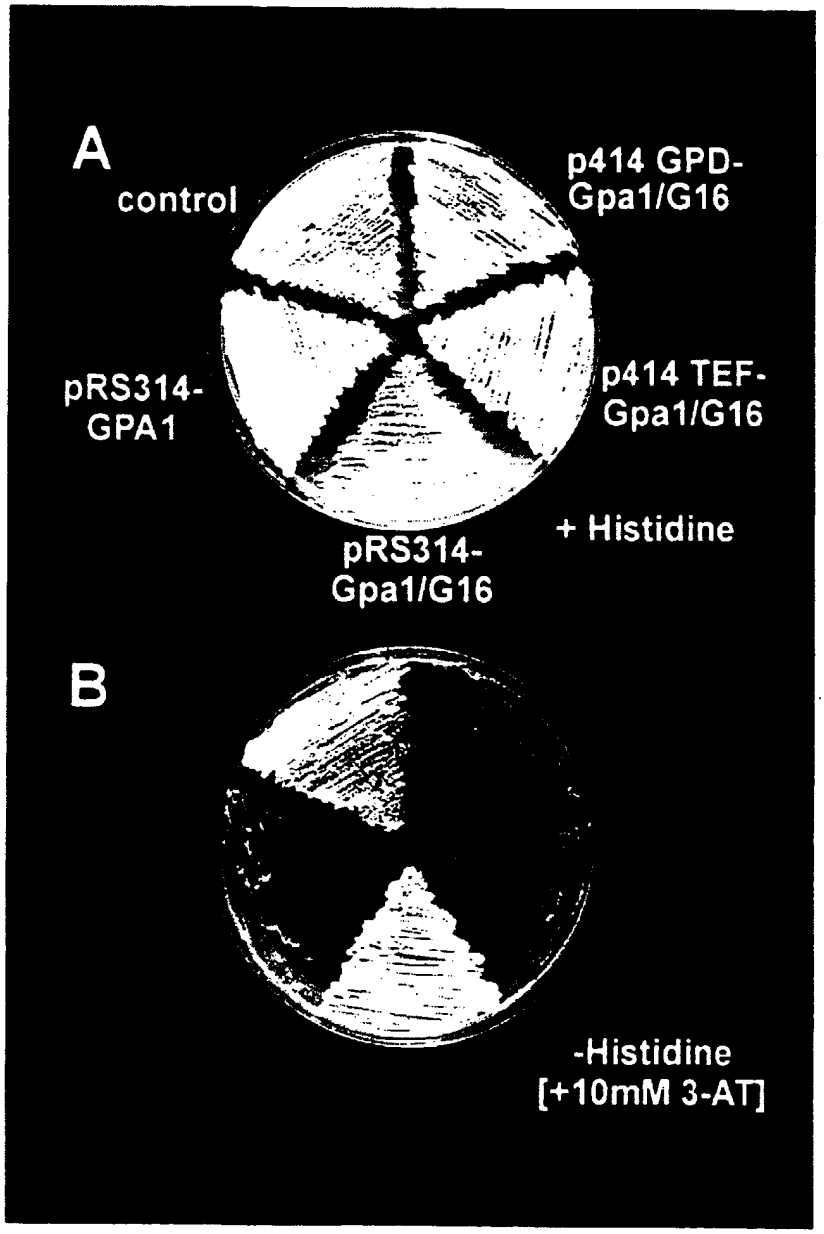

A
control
p414 GPD-Gpa1/G16
pRS314-GPA1
p414 TEF-Gpa1/G16
+ Histidine
pRS314-Gpa1/G16
B
-Histidine
[+10mM 3-AT]

FIG.3

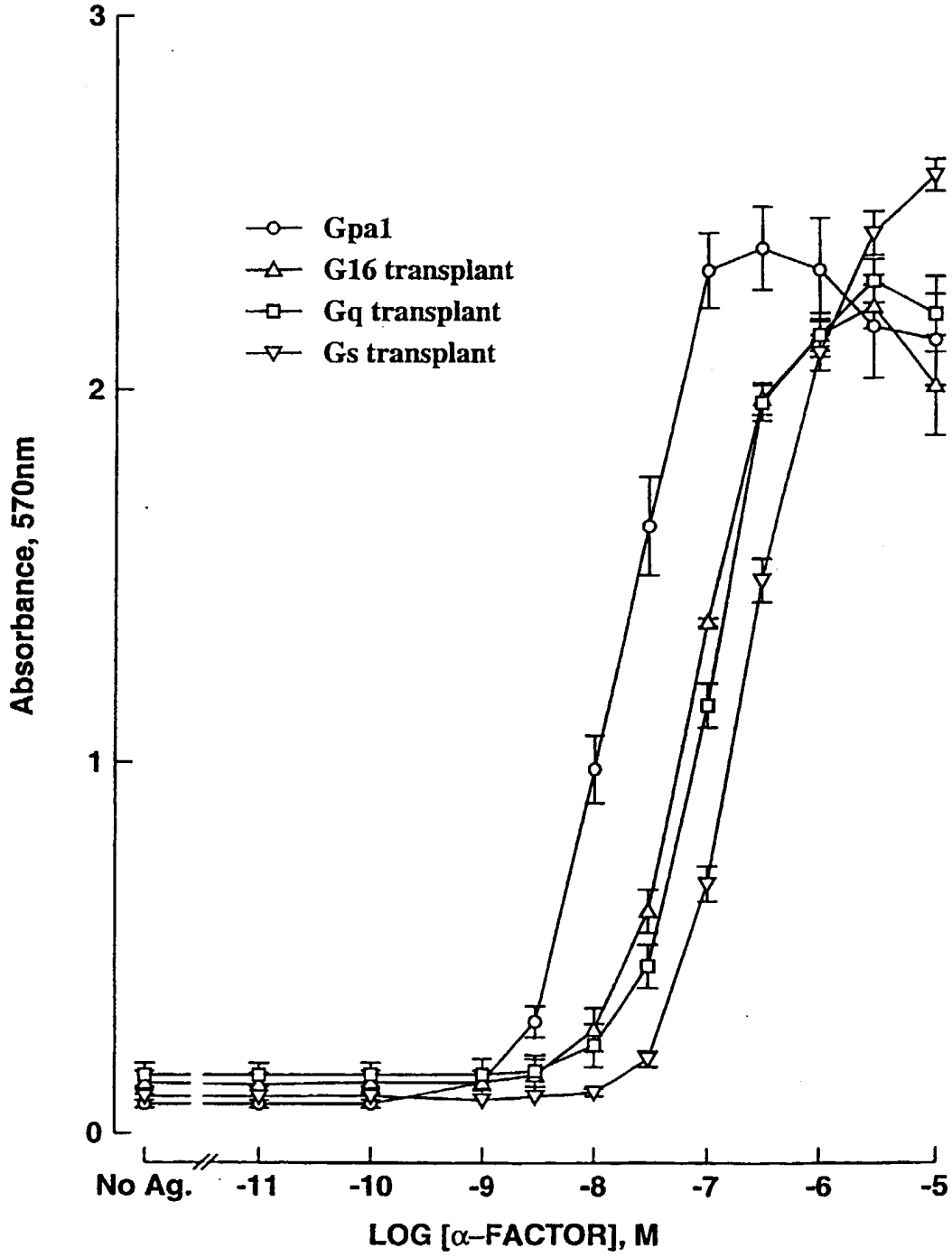

Ste2 Receptor
Gpa1
G16 transplant
Gq transplant
Gs transplant
Absorbance, 570nm
3
2
1
0
No Ag.
-11
-10
-9
-8
-7
-6
-5
LOG [α–FACTOR], M

FIG.4(a)

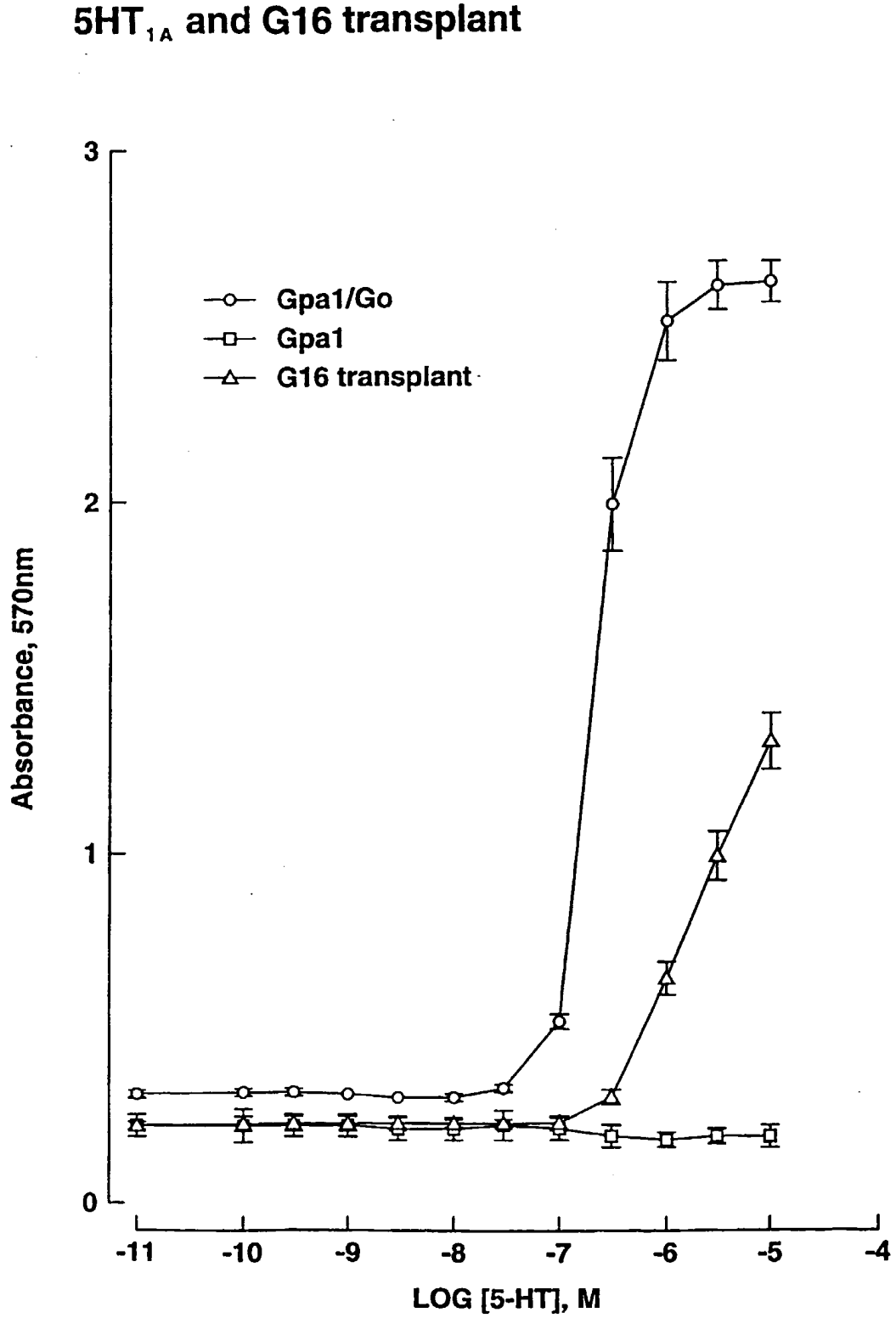

5HT1A and G16 transplant
Gpa1/Go
Gpa1
G16 transplant
Absorbance, 570nm
0
1
2
3
-11
-10
-9
-8
-7
-6
-5
-4
LOG [5-HT], M

FIG. 4(b)

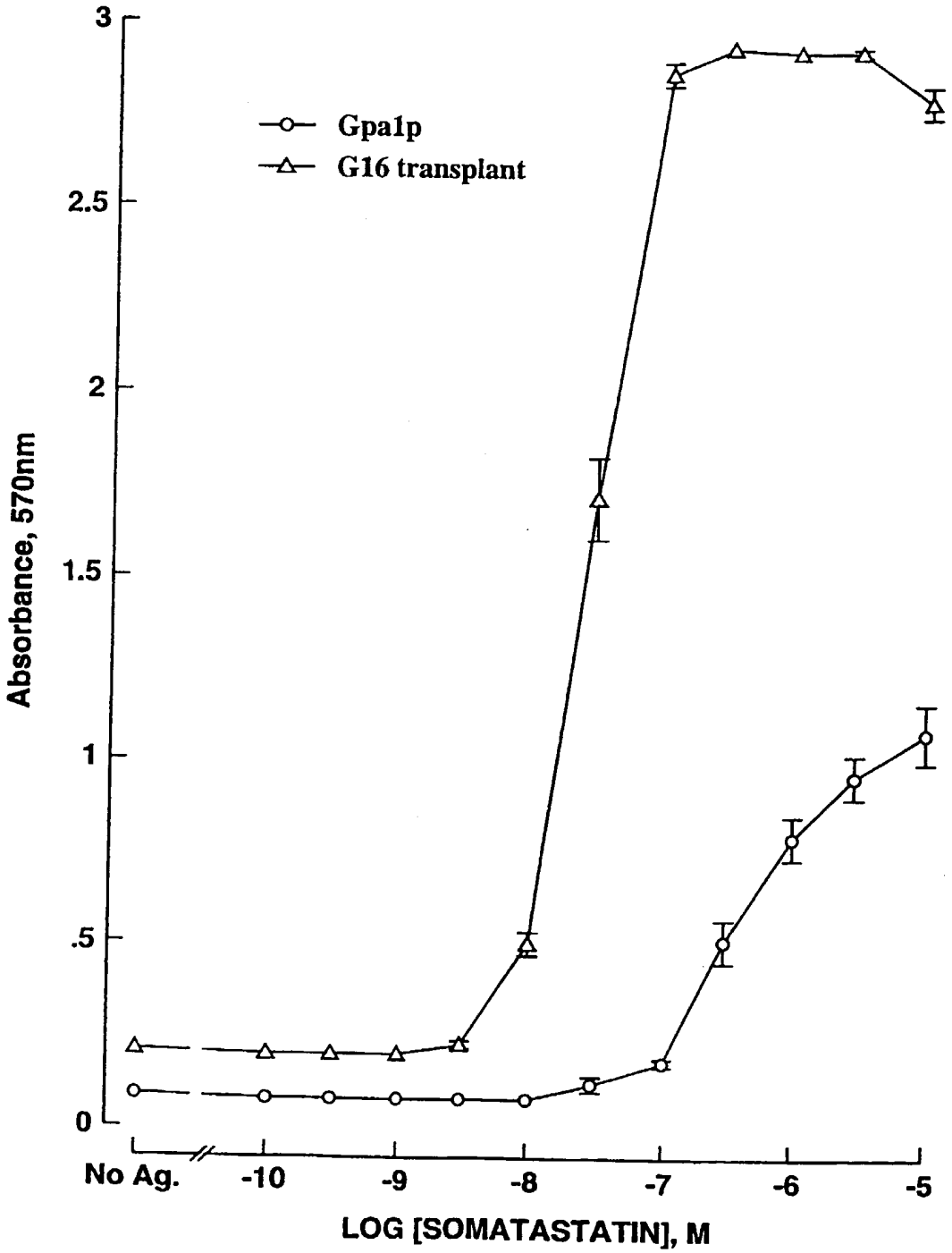

SST2 Receptor
Gpa1p
G16 transplant
Absorbance, 570nm
3
2.5
2
1.5
1
.5
0
No Ag.
-10
-9
-8
-7
-6
-5
LOG [SOMATASTATIN], M

FIG.4(c)

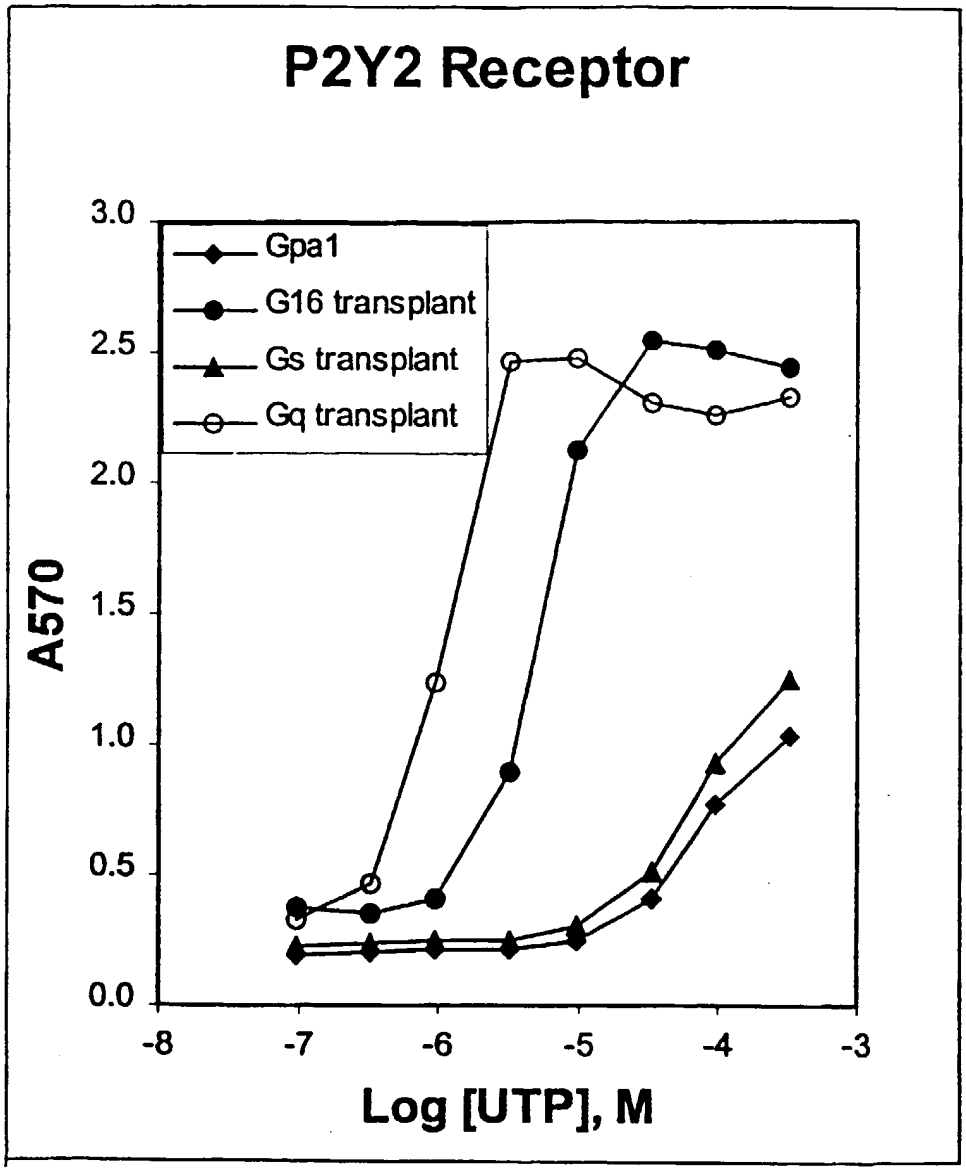

P2Y2 Receptor
Gpa1
G16 transplant
Gs transplant
Gq transplant
A570
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-8
-7
-6
-5
-4
-3
Log [UTP], M

FIG.4(d)

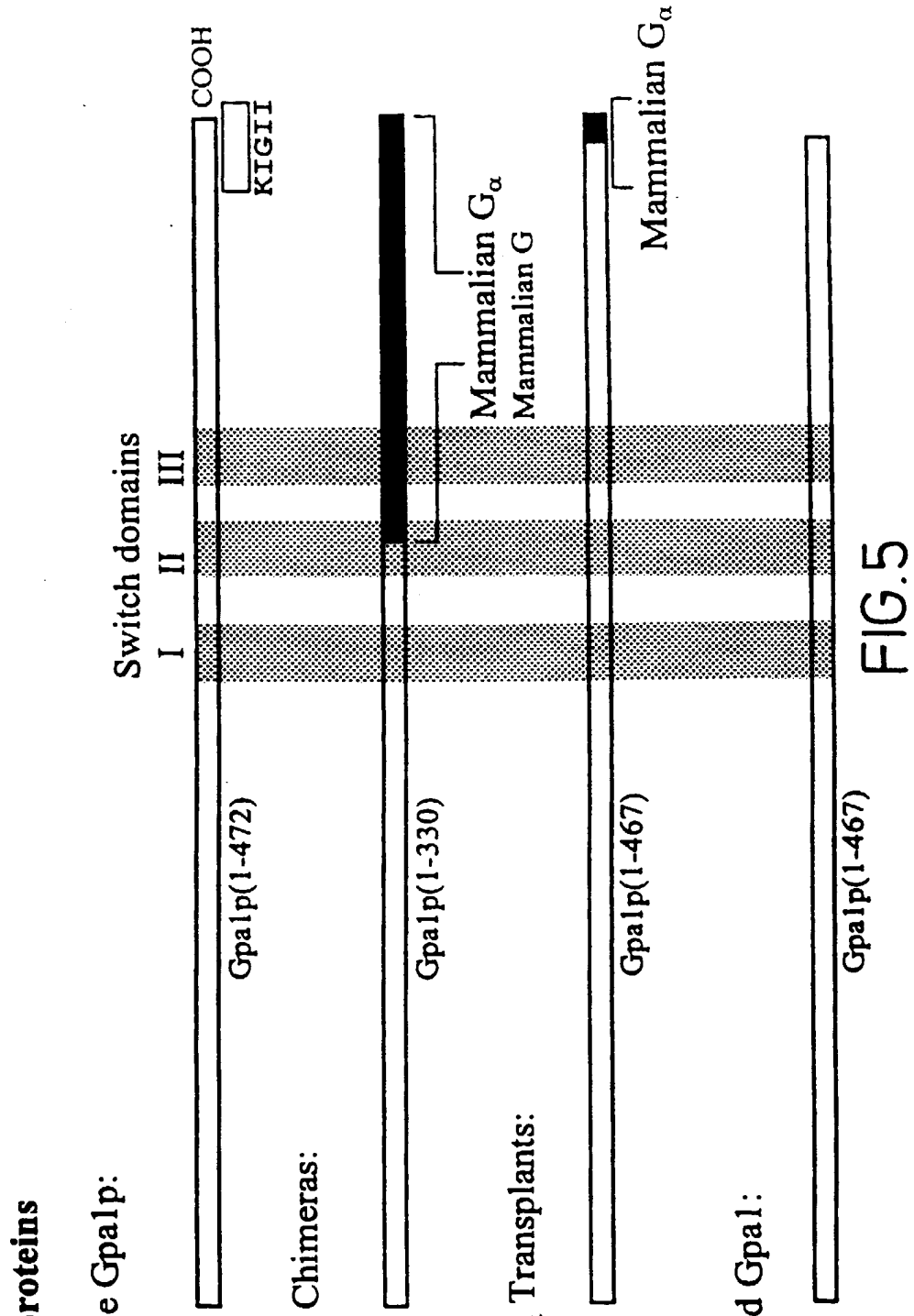

Gα proteins
A. Wild-type Gpa1p:
B. Gpa1/Gα Chimeras:
C. Gpa1/Gα Transplants:
D. Truncated Gpa1:
Switch domains
I II III
NH2
COOH
KIGII
Gpa1p(1-472)
Gpa1p(1-330)
Mammalian Gα
Mammalian G
Gpa1p(1-467)
Mammalian Gα
Gpa1p(1-467)

FIG.5

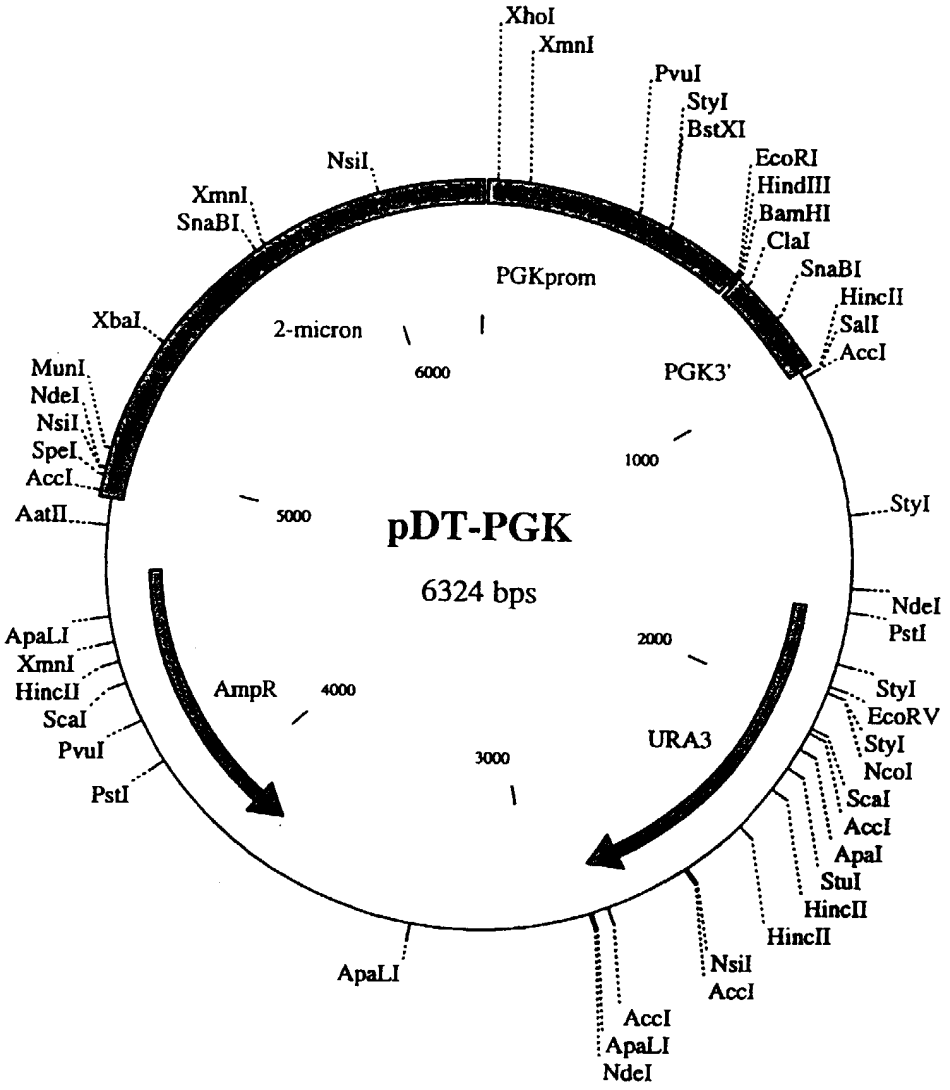

XhoI
XmnI
PvuI
StyI
BstXI
EcoRI
HindIII
BamHI
ClaI
SnaBI
HincII
SalI
AccI
StyI
NdeI
PstI
StyI
EcoRV
StyI
NcoI
ScaI
AccI
ApaI
StuI
HincII
HincII
NsiI
AccI
AccI
ApaLI
NdeI
ApaLI
PstI
PvuI
ScaI
HincII
XmnI
ApaLI
AatII
AccI
SpeI
NsiI
NdeI
MunI
XbaI
XmnI
SnaBI
NsiI
PGKprom
PGK3'
URA3
AmpR
2-micron
pDT-PGK
6324 bps
1000
2000
3000
4000
5000
6000

FIG.6(a)

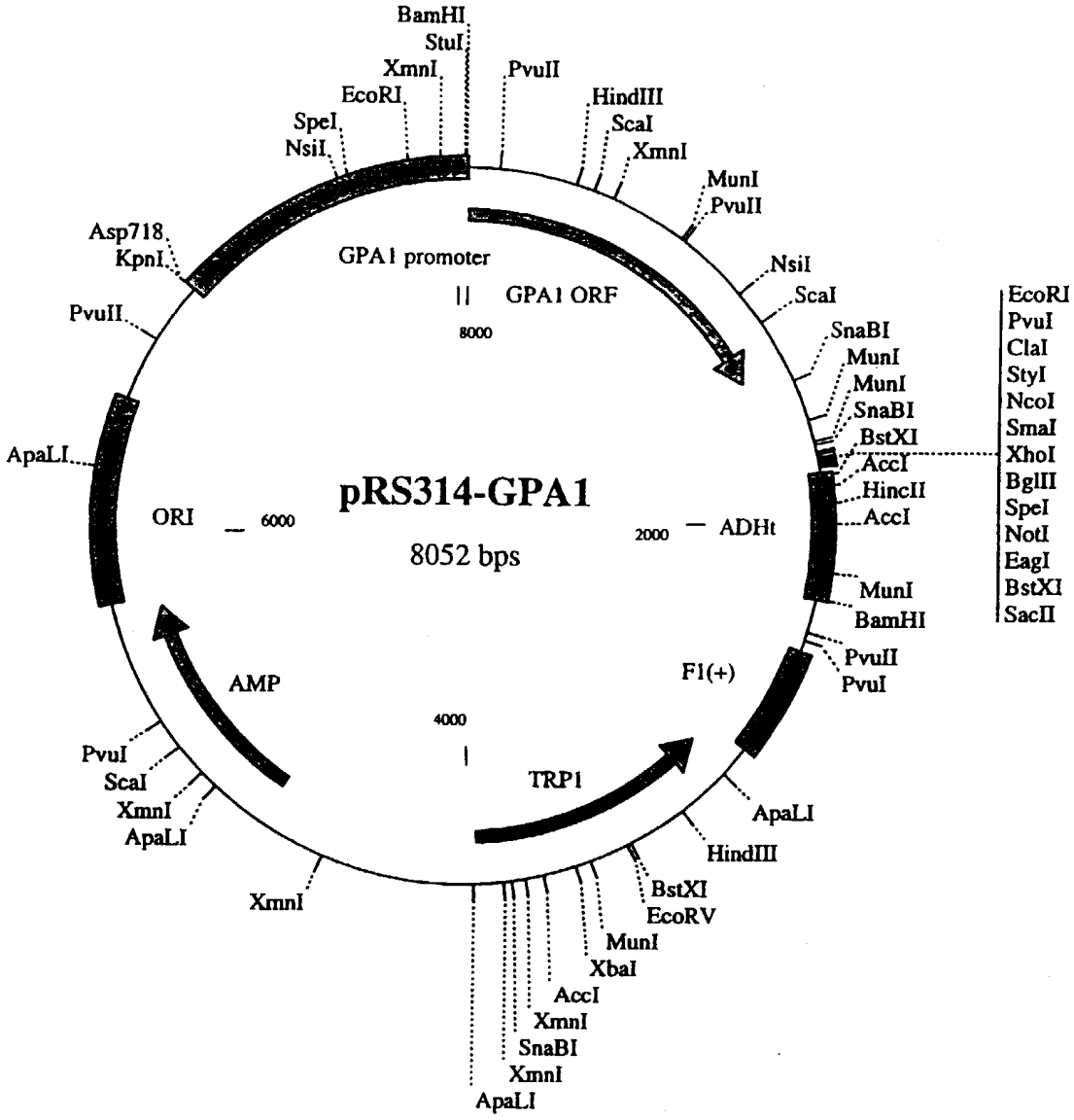

pRS314-GPA1
8052 bps
GPA1 promoter
GPA1 ORF
ADHt
TRP1
F1(+)
AMP
ORI
2000
4000
6000
8000
BamHI
StuI
XmnI
EcoRI
SpeI
NsiI
PvuII
HindIII
ScaI
XmnI
MunI
PvuII
NsiI
ScaI
SnaBI
MunI
MunI
SnaBI
BstXI
AccI
HincII
AccI
MunI
BamHI
PvuII
PvuI
ApaLI
HindIII
BstXI
EcoRV
MunI
XbaI
AccI
XmnI
SnaBI
XmnI
ApaLI
XmnI
PvuI
ScaI
XmnI
ApaLI
ApaLI
PvuII
Asp718
KpnI
EcoRI
PvuI
ClaI
StyI
NcoI
SmaI
XhoI
BglII
SpeI
NotI
EagI
BstXI
SacII

FIG. 6(c)

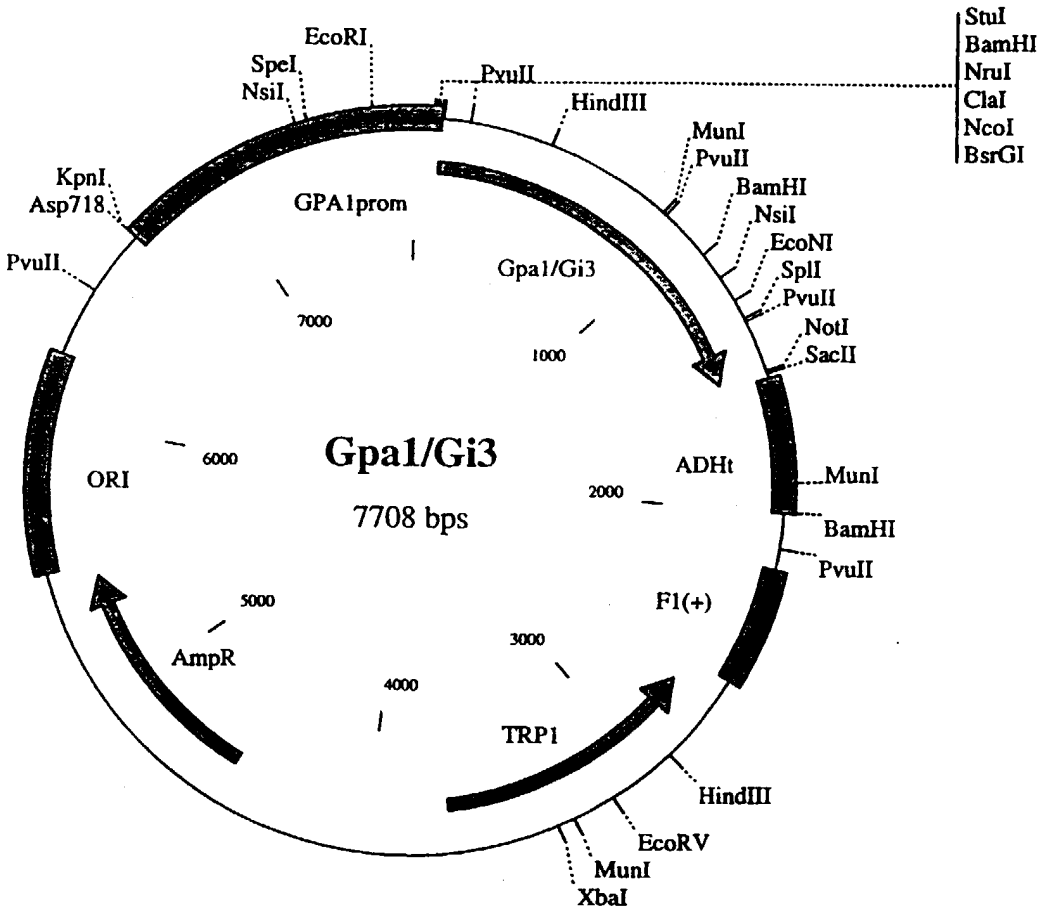

Gpa1/Gi3
7708 bps
GPA1prom
Gpa1/Gi3
ADHt
F1(+)
TRP1
AmpR
ORI
1000
2000
3000
4000
5000
6000
7000
EcoRI
SpeI
NsiI
PvuII
HindIII
MunI
PvuII
BamHI
NsiI
EcoNI
SplI
PvuII
NotI
SacII
MunI
BamHI
PvuII
HindIII
EcoRV
MunI
XbaI
PvuII
KpnI
Asp718
StuI
BamHI
NruI
ClaI
NcoI
BsrGI

FIG.6(d)

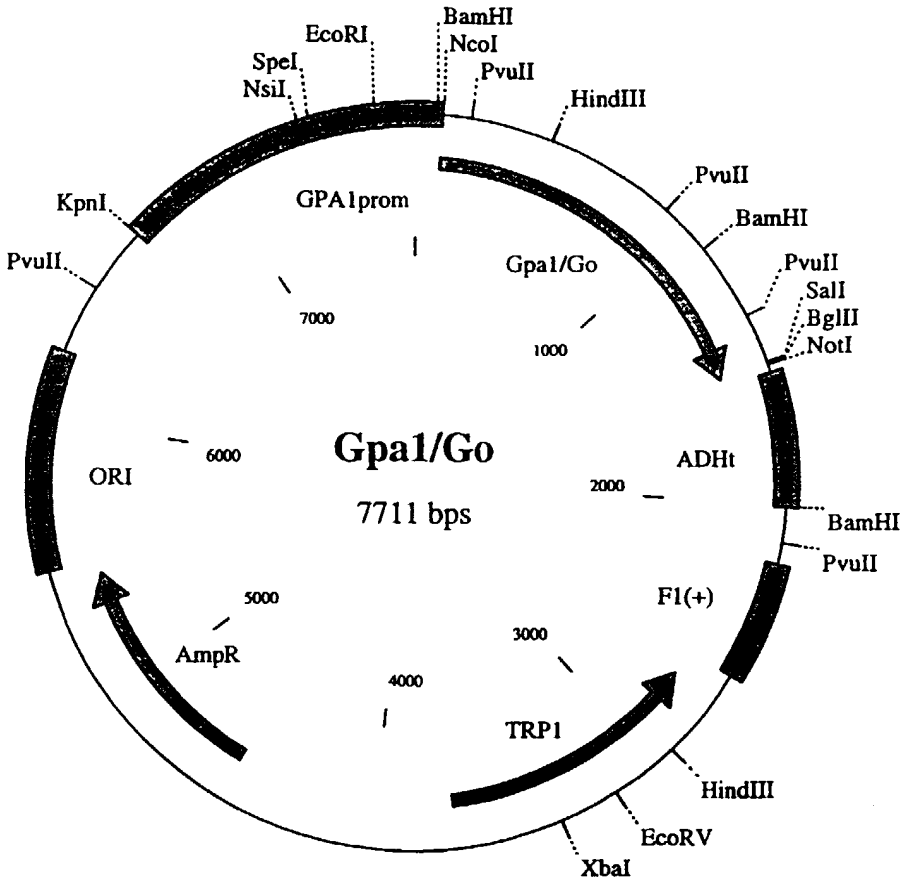

BamHI
NcoI
EcoRI
SpeI
NsiI
PvuII
HindIII
PvuII
BamHI
PvuII
SalI
BglII
NotI
KpnI
PvuII
GPA1prom
Gpa1/Go
7000
1000
Gpa1/Go
7711 bps
6000
ORI
ADHt
2000
BamHI
PvuII
5000
AmpR
F1(+)
3000
4000
TRP1
HindIII
EcoRV
XbaI

FIG.6(e)

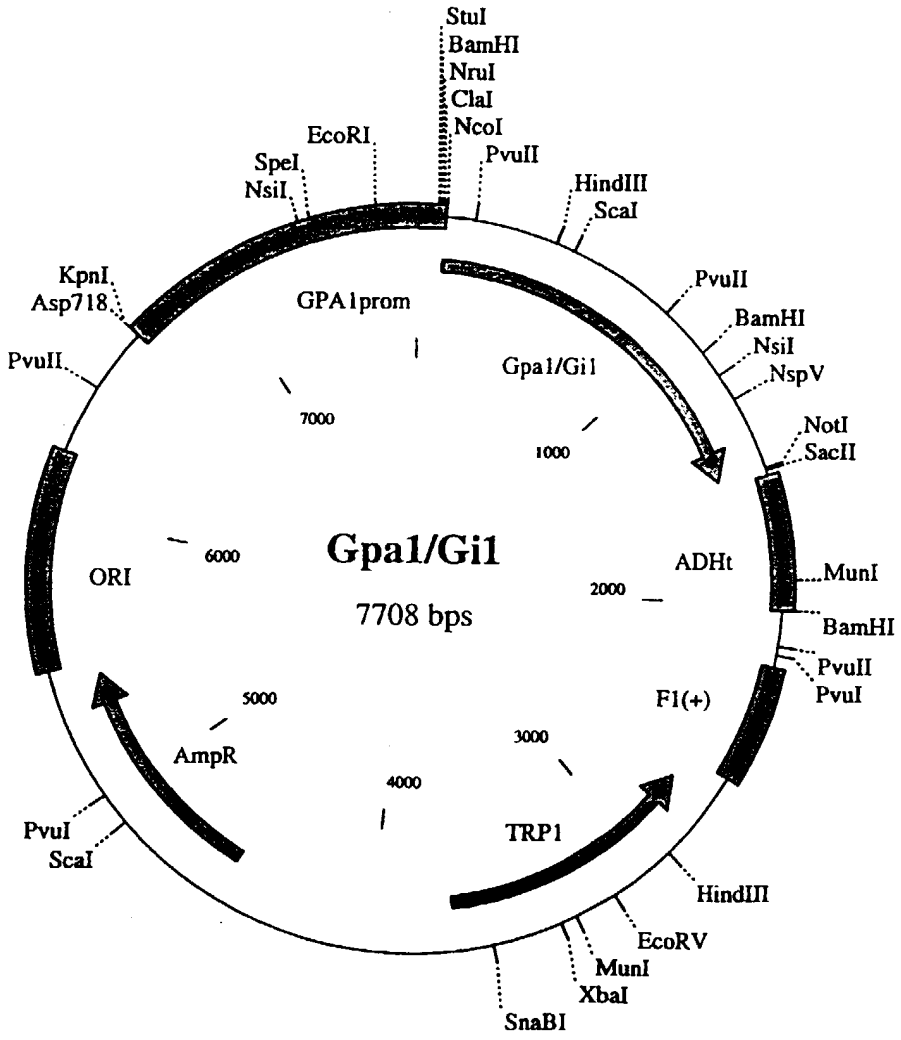

StuI
BamHI
NruI
ClaI
NcoI
PvuII
HindIII
ScaI
PvuII
BamHI
NsiI
NspV
NotI
SacII
MunI
BamHI
PvuII
PvuI
F1(+)
HindIII
EcoRV
MunI
XbaI
SnaBI
TRP1
PvuI
ScaI
AmpR
ORI
PvuII
KpnI
Asp718
SpeI
NsiI
EcoRI
GPA1prom
Gpa1/Gi1
ADHt
Gpa1/Gi1
7708 bps
1000
2000
3000
4000
5000
6000
7000

FIG.6(f)

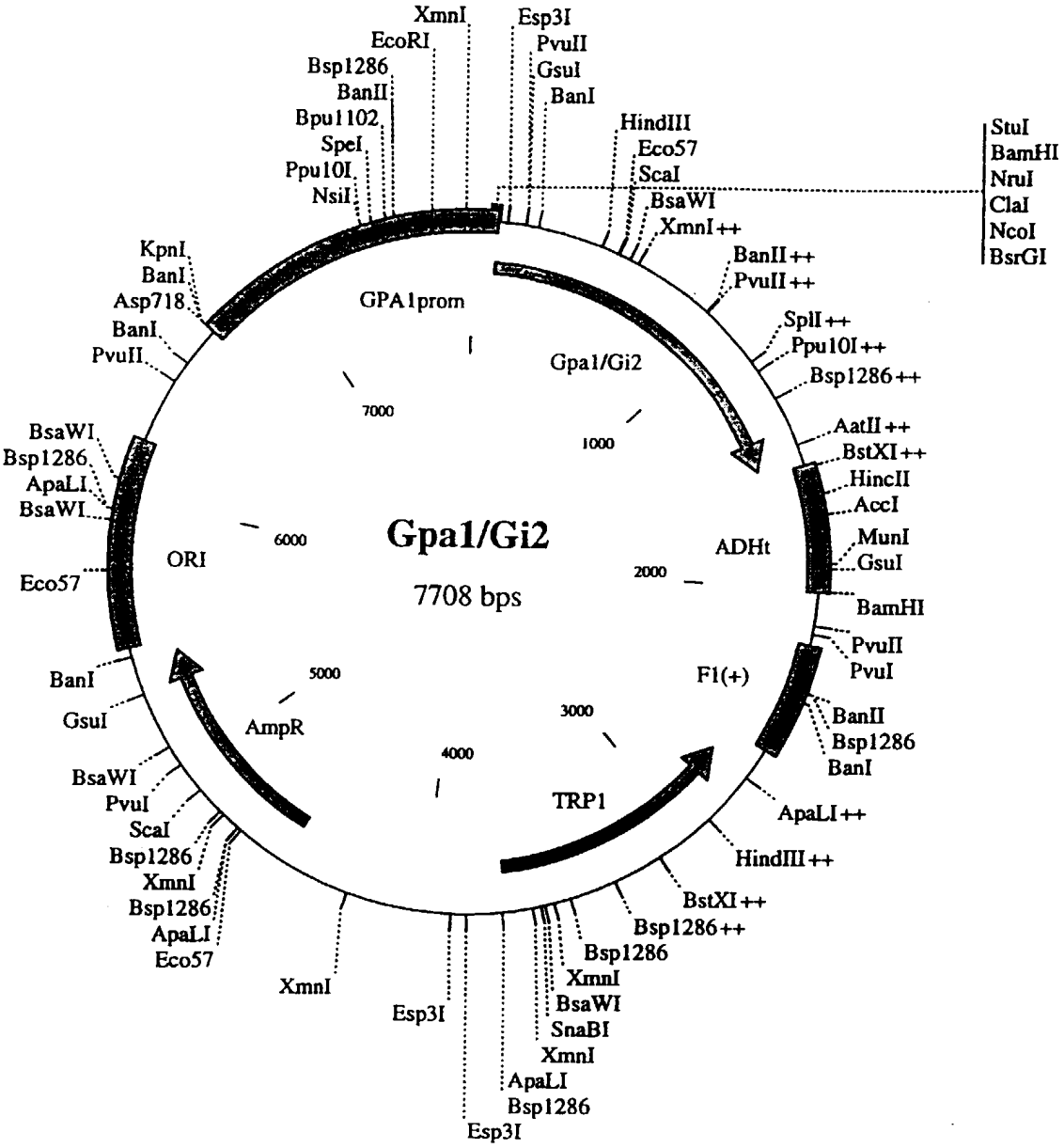

Gpa1/Gi2
7708 bps
GPA1prom
Gpa1/Gi2
ADHt
F1(+)
TRP1
AmpR
ORI
1000
2000
3000
4000
5000
6000
7000
XmnI
EcoRI
Bsp1286
BanII
Bpu1102
SpeI
Ppu10I
NsiI
Esp3I
PvuII
GsuI
BanI
HindIII
Eco57
ScaI
BsaWI
XmnI++
BanII++
PvuII++
SpII++
Ppu10I++
Bsp1286++
AatII++
BstXI++
HincII
AccI
MunI
GsuI
BamHI
PvuII
PvuI
BanII
Bsp1286
BanI
ApaLI++
HindIII++
BstXI++
Bsp1286++
Bsp1286
XmnI
BsaWI
SnaBI
XmnI
ApaLI
Bsp1286
Esp3I
XmnI
Esp3I
ApaLI
Eco57
Bsp1286
XmnI
Bsp1286
ScaI
PvuI
BsaWI
GsuI
BanI
Eco57
BsaWI
ApaLI
Bsp1286
BsaWI
PvuII
BanI
Asp718
BanI
KpnI
StuI
BamHI
NruI
ClaI
NcoI
BsrGI

FIG.6(g)

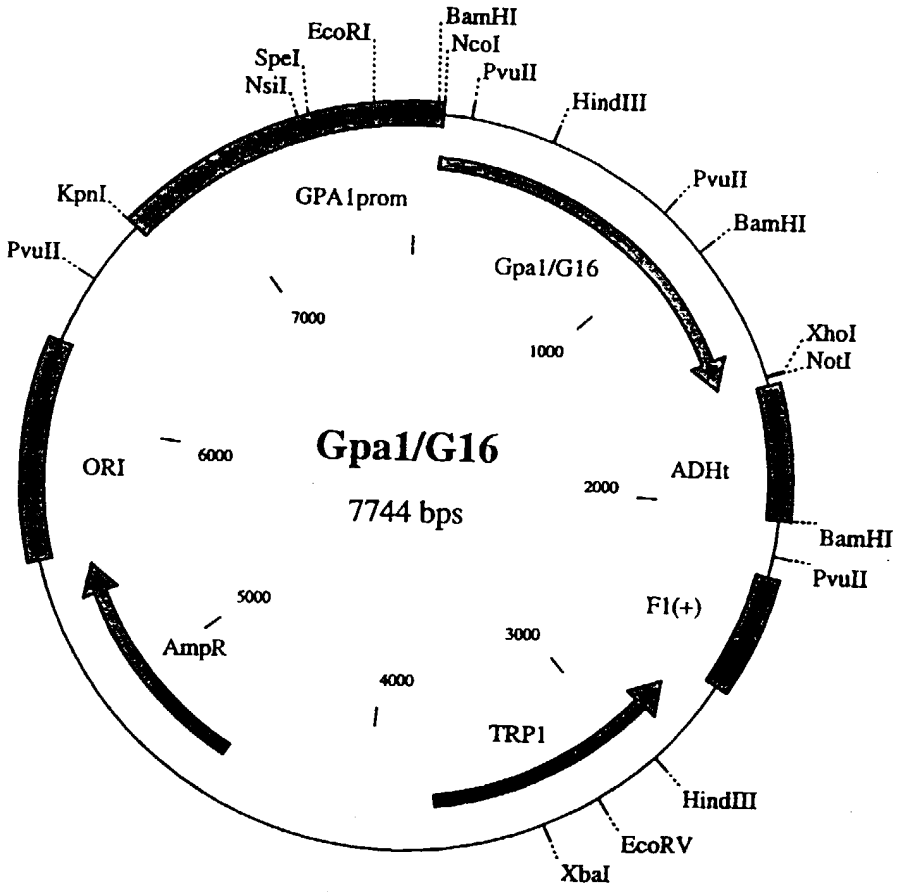

BamHI
NcoI
EcoRI
SpeI
NsiI
PvuII
HindIII
PvuII
BamHI
XhoI
NotI
KpnI
PvuII
GPA1prom
Gpa1/G16
7000
1000
Gpa1/G16
7744 bps
ORI
6000
ADHt
2000
BamHI
PvuII
F1(+)
5000
AmpR
3000
4000
TRP1
HindIII
EcoRV
XbaI

FIG. 6(h)

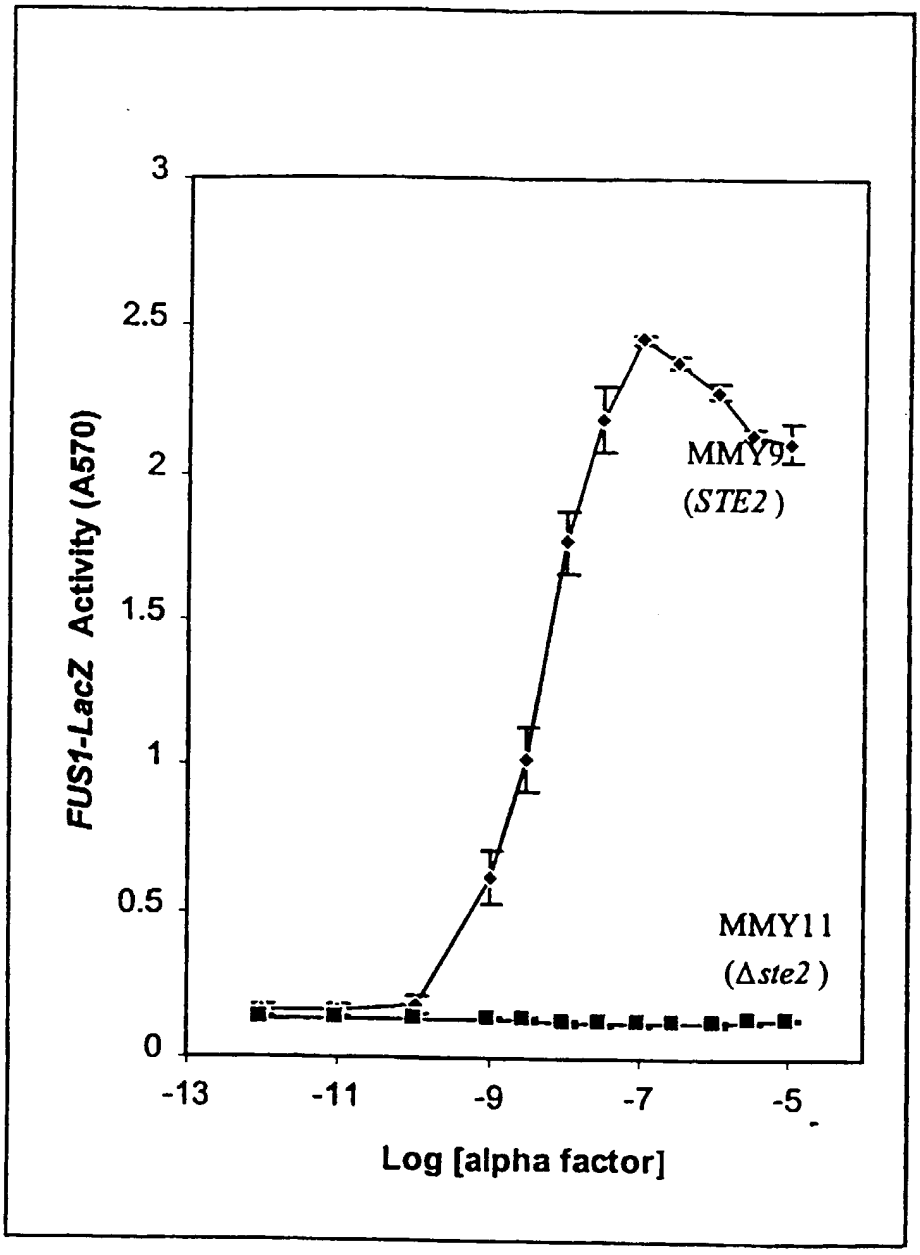

3
2.5
2
1.5
1
0.5
0
FUS1-LacZ Activity (A570)
-13
-11
-9
-7
-5
Log [alpha factor]
MMY9
(STE2)
MMY11
(Δste2)

FIG.7

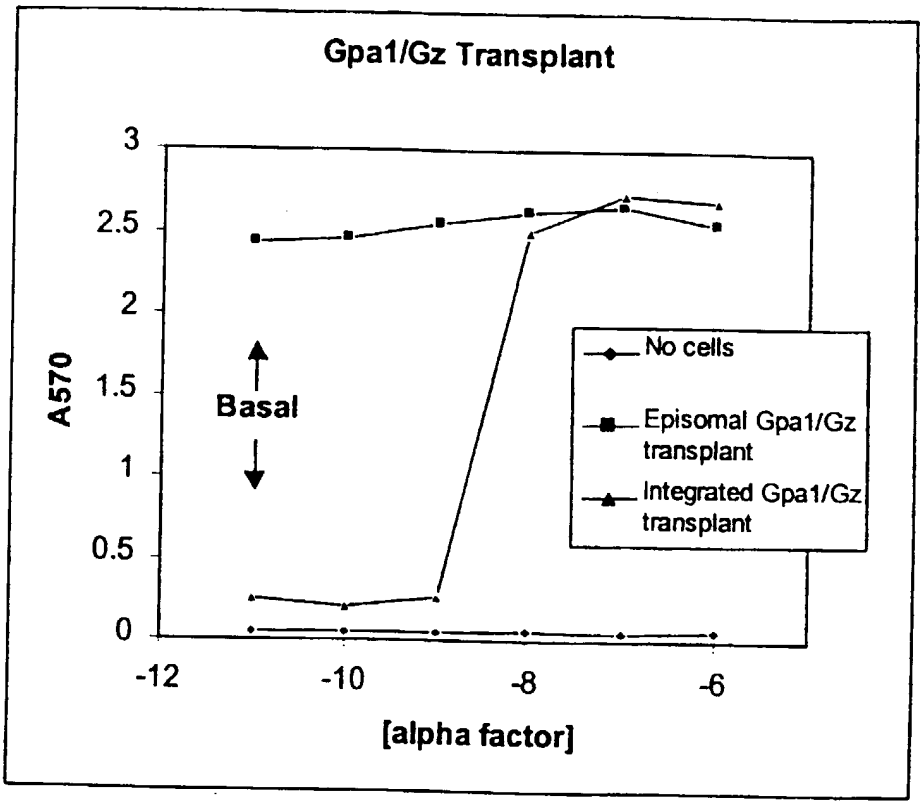

Gpa1/Gz Transplant
3
2.5
2
1.5
1
0.5
0
A570
Basal
No cells
Episomal Gpa1/Gz transplant
Integrated Gpa1/Gz transplant
-12
-10
-8
-6
[alpha factor]

FIG.8(a)

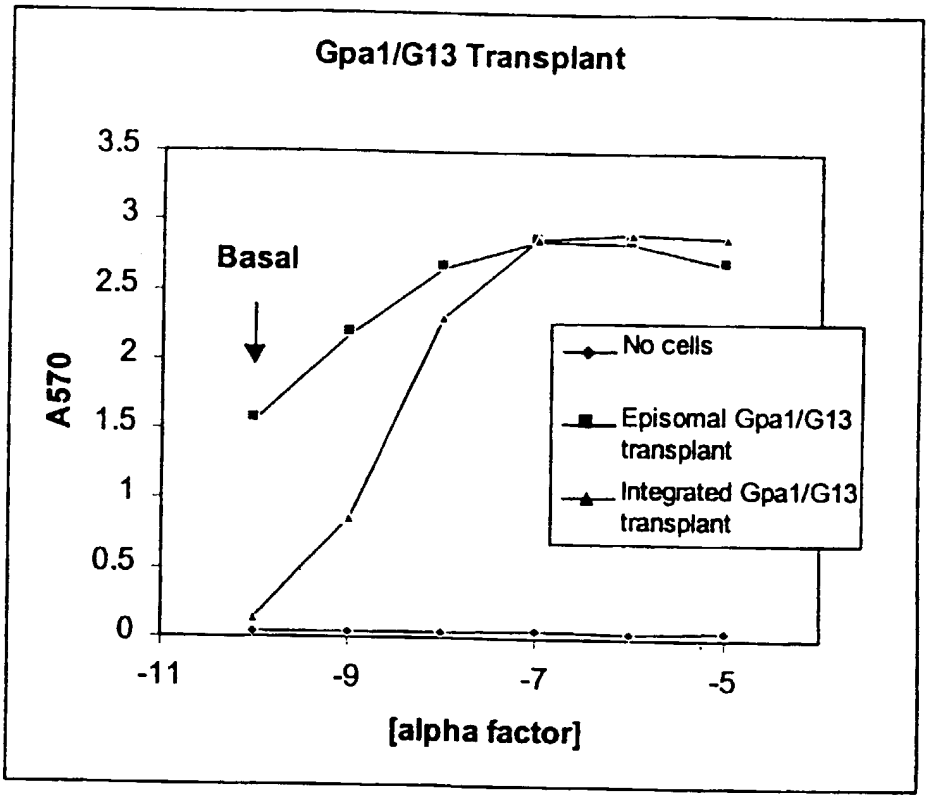

Gpa1/G13 Transplant
3.5
3
2.5
2
1.5
1
0.5
0
A570
Basal
No cells
Episomal Gpa1/G13 transplant
Integrated Gpa1/G13 transplant
-11
-9
-7
-5
[alpha factor]

FIG.8(b)

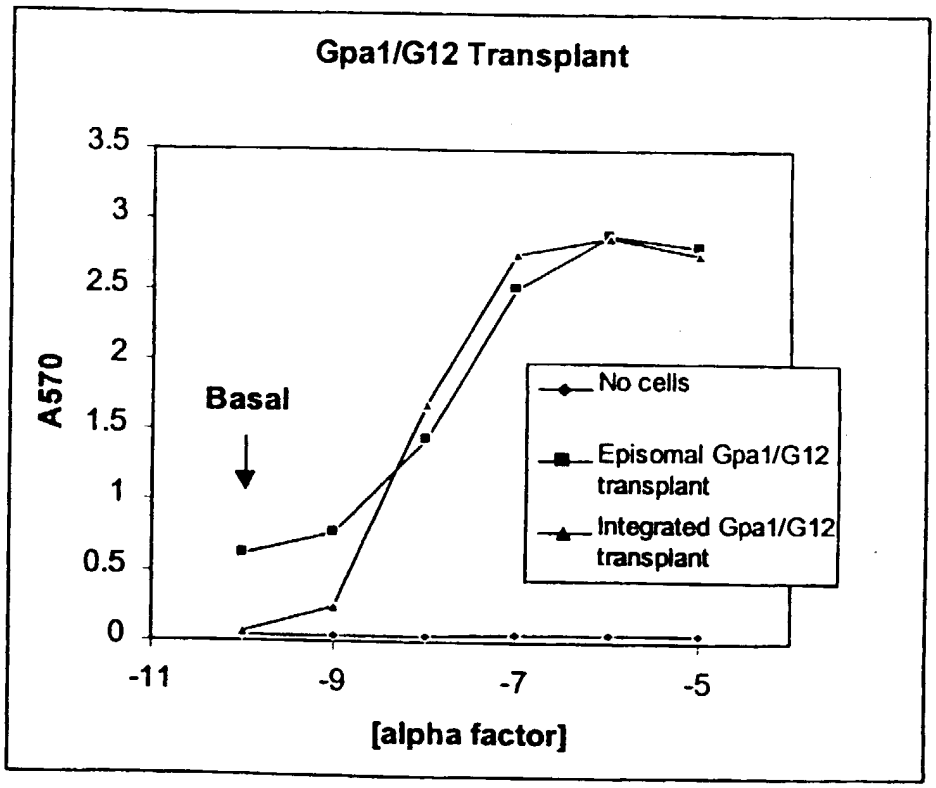

Gpa1/G12 Transplant
3.5
3
2.5
2
1.5
1
0.5
0
A570
Basal
-11
-9
-7
-5
[alpha factor]
No cells
Episomal Gpa1/G12 transplant
Integrated Gpa1/G12 transplant

FIG.8(c)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/GB 98/02759

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 16557 A (GEN HOSPITAL CORP) 23 April 1998 see page 3 - page 7 see figure 1 see claims 1-27	1-8

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/GB 98/02759

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9521925 A	17-08-1995	US 5691188 A	25-11-1997
		AU 1846995 A	29-08-1995
		CA 2183166 A	17-08-1995
		EP 0745130 A	04-12-1996
		JP 9510087 T	14-10-1997
		SG 49061 A	18-05-1998
		US 5846819 A	08-12-1998
WO 9816557 A	23-04-1998	NONE	

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 98/02759

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C12N1/19 C07K14/47 C12Q1/68 G01N33/50
//C07K14/72,C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 21925 A (AMERICAN CYANAMID CO ;PAUSCH MARK HENRY (US); OZENBERGER BRADLEY A) 17 August 1995 cited in the application see abstract see claims 3,4,17,18,22,27	1-8
A	CONKLIN B.R: ET AL.: "Carboxyl-terminal mutations of Gq alpha and Gs alpha that alter the fidelity of receptor activation" MOLECULAR PHARMACOLOGY, vol. 50, no. 4, October 1996, pages 885-890, XP002090508 see the whole document	1-8

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 January 1999	03/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Galli, I

2

Form PCT/ISA/210 (second sheet) (July 1992)